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Genetic and phenotypic characterisation of the *Cftr*$^{TgH(neoim)Hgu}$ cystic fibrosis mouse models

Mausmodelle für die zystische Fibrose: Geno-und phänotypische Charakterisierung der *Cftr*$^{TgH(neoim)Hgu}$ Mäusestämme

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To my parents.

(In Greek) Στους γονείς μου.

„Whoever you are, or whateverit is that you do, when you really want something it is because that desire originated in the soul of the universe, and so when you want something, all the universe conspires in helping you to achieve it."

Paulo Coelho
"The Alchemist"

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Abstract

Cystic fibrosis (CF) is an autosomal recessive disease, caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a phosphorylation –regulated Cl^- channel expressed in the apical membrane of epithelia, and is characterised by pathological features of variable severity. Isolation of the murine homologue (*Cftr*) of the human gene enabled the development of transgenic cystic fibrosis mouse models. Using insertional mutagenesis in *Cftr* exon 10 Dorin *et al.*, 1992 developed the C*ftr*$^{TgH(neoim)Hgu}$ mouse model of CF. A pair of homozygous C*ftr*$^{TgH(neoim)Hgu}$ animals with a mixed genetic background became the founders for the generation of two inbred C*ftr*$^{TgH(neoim)Hgu}$ strains CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$. In the context of this thesis these two inbred CF strains were both genetically and phenotypically characterised. Biochemical and functional analysis of the two strains revealed that, unlike their progenitors with a mixed genetic background, trace amounts of correctly spliced mRNA and subsequent production of low amounts of Cftr protein (10-20%) substantiated for the amelioration of the basic defect in the nose and the intestine with the presentation of a subnormal phenotype when compared to wild type and F508del homozygous animals. In order to dissect the role of the mutation from the genetic background the C*ftr*$^{TgH(neoim)Hgu}$ was backcrossed to three different inbred genetic backgrounds (C57BL/6, BALB/c, DBA/2J). The demand of a high throughput identification of animals carrying the insertional mutation led to the establishment of a PCR based protocol whereby the Southern RFLP was replaced with intragenic *Cftr* microsatellite markers directly linked with the disrupted locus in exon 10. This alternative method assisted in the identification of unexpected events such as *Cftr* intragenic recombination between the donor and the recipient allele and construct excision. Microsatellite markers also provided a useful tool for the genetic characterisation of the two inbred CF strains and the three inbred wild type strains aiming to identify the genetic relationship of the strains before the phenotypic characterisation of the congenic C*ftr*$^{TgH(neoim)Hgu}$ animals allowing for future linkage studies which will permit the mapping of modifiers of this cystic fibrosis phenotype.

Key Words: cystic fibrosis, mouse model, microsattelite genome scan.

Abstrakt

Cystische Fibrose (CF) ist eine autosomal rezessive Vererbte und hinsichtlich des Schweregrades der Pathologie stark variable Erkrankung, die durch Mutationen im *Cystic Fibrosis Transmembrane Conductance Regulator* – Gen (*CFTR*) ausgelöst wird. Das *CFTR*-Gen kodiert für einen durch Phosphorylierung regulierten Chloridkanal, der in der Apikalmembran epithelialer Zellen exprimiert wird. Die Beschreibung des murinen Homologs (*Cftr*) des menschlichen *CFTR*-Gens hat es ermöglicht, transgene Mäuse als Tiermodell der CF zu entwickeln. Durch Insertionsmutagenese im Exon 10 des *Cftr*-Gens haben Dorin *et al*., 1992 das C*ftr*$^{TgH(neoim)Hgu}$ Mausmodell der CF entwickelt. Aus einem Paar homozygoter C*ftr*$^{TgH(neoim)Hgu}$ Tiere mit gemischtem genetischen Hintergrund wurden die Inzuchtstämme CF/1- C*ftr*$^{TgH(neoim)Hgu}$ und CF/3- C*ftr*$^{TgH(neoim)Hgu}$gezüchtet. Im Rahmen dieser Arbeit wurden diese beiden CF Inzuchtstämme genetisch und phänotypisch charakterisiert. Biochemische und funktionelle Analysen der beiden Inzuchtstämme haben gezeigt, daß diese Inzuchtstämme — im Gegensatz zu ihren Vorfahren mit gemischtem genetischen Hintergrund — Spuren von regelgerecht prozessierter Cftr-mRNA bilden und nachfolgend geringe Mengen von Cftr-Protein (10-20%) exprimieren. Diese Menge des Cftr-Proteins stellt die Basis für die bei den Tieren beobachtete Verbesserung des Basisdefektes in nasalem und intestinalem Gewebe dar, wobei sich der Phänotyp der Inzuchtstämme im Vergleich zu Wildtyp-Tieren und zu F508del homozygoten CF Mäusen als subnormal darstellt. Um den relativen Einfluß der Mutation von dem des genetischen Hintergrundes zu unterscheiden, wurden die C*ftr*$^{TgH(neoim)Hgu}$ Mäuse in drei verschiedene Inzuchtstämme rückgekreuzt (C57BL/6, BALB/c, DBA/2J). Für diese Kreuzungsexperimente mußte ein Hochdurchsatzverfahren entwickelt werden, das die Identifikation der Tiere erlaubt, die die Insertionsmutation tragen. Zu diesem Zweck wurde das bislang genutzte zeitaufwendige Verfahren der Southernblot-RFLP-Typisierung durch die Typisierung mit intragenischer Mikrosatelliten, die im Kopplungsungleichgewicht mit dem unterbrochenen Exon 10 liegen, substituiert. Diese alternative Methode erlaubte es, unerwartete Ereignisse während der Zucht — wie die intragenische Rekombination zwischen Wildtyp-Allel und CF-Allel sowie die Exzision des Insertionskonstruktes — zu erkennen und zu dokumentieren. Mikrosatellitenmarker wurden desweiteren eingesetzt, um die beiden CF Inzuchtstämme und die drei Wildtyp-Inzuchtstämme genetisch zu charakterisieren. Diese Daten dienen als Grundlage für zukünftige Kopplungsanalysen, die die Kartierung von Modulatoren des CF Phänotyps in den verschiedenen CF-Mäuselinien Charakterisierung ermöglichen werden.

Schlagwörter: Cystische Fibrose, Mausmodell, Microsatelliten.

Chapter 1

Mouse Models Of Cystic Fibrosis-General Introduction.

1.1) Cystic Fibrosis

The autosomal recessive disease Cystic Fibrosis (CF) was first recognised as an entity by Fanconi in 1936. Andersen gave the first detailed description of the disease and called it "cystic fibrosis of the pancreas". Farber (1945) introduced the term, "mucoviscidosis" which became popular in North America, but "cystic fibrosis" became the term most frequently used. The cloning, in 1989, of the gene responsible for the disease- the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan *et al*., 1989, Rommens *et al*., 1989, Kerem *et al*., 1989)- sparked understanding of the genetics of CF. Located on the long arm of chromosome 7, it spans over 250kb, contains 27 exons and encodes a 1480 residue transmembrane glycoprotein. The protein consists of two repeated motifs made up of six membrane spanning segments, and a nucleotide binding domain (NBD) separated by a regulatory domain (R domain) with multiple potential phosphorylation sites.

CFTR is being highly expressed in the epithelial cells of the sweat ducts, the pancreatic ducts, the digestive tract, particularly in the crypts of the small intestine and Brunner's glands, the biliary ducts, the salivary glands, the reproductive organs and the lungs, particularly in the serous cells of the submucosal glands and in the respiratory epithelial cells (Davis *et al*., 1996). Characteristic manifestations of the CF disease are primarily due to the dysfunction of exocrine glands resulting in the production of viscus dehydrated secretions. The resulting phenotype is salty sweat, pancreatic insufficiency, intestinal obstruction with meconium ileus (MI) in 10-20% few days after birth, infertility and severe pulmonary disease (Anderson *et al*., 1992). CFTR functions as a cAMP- regulated chloride ion channel at the apical membrane of epithelial cells, and the electrophysiological profile of affected tissues shows characteristic abnormalities in the transport of chloride and sodium ions across the apical membrane. Palmonary disease is the major cause of morbidity and mortality in CF, patients develop chronic bacterial infection, abnormal airway secretions and airway inflammation, resulting in progressive bronchiectasis, respiratory failure and death (Davidson *et al*., 1998).

1.2) Generation of mouse models of cystic fibrosis

1.2.1) The murine CF gene.

The murine homologue of the human *CFTR* gene on chromosome 6 was isolated in 1991 (Tata *et al*., 1991). The *Cftr* gene spans 152kb, sequencing and comparative analysis with the human *CFTR* gene (Ellsworth *et al*., 2000), revealed high similarity between the 27 exons with the human homologue. Also on the protein level the murine CFTR protein displays a 78% overall sequence identity at the amino acid level with the majority of known CF mutations occurring in conserved regions (Dorin *et al*., 1994a).

1.2.2) Mouse models

To date thirteen CF mouse models have been described in the literature. For the creation of the CF mouse models the murine *Cftr* gene is mutated in the desired fashion and cloned into a targeting vector which is then inserted into murine embryonic stem cells (Robertson *et al*., 1986, Gossler *et al*., 1986, Doetschman *et al*., 1986). The mutated gene integrates into the homologous gene locus of the stem cells via homologous recombination (Smithies *et al*., 1985, Thomas *et al*., 1987). Correctly targeted stem cells are isolated, expanded, injected into murine blastocysts, and transferred to a pseudo-pregnant foster mother. This produces a chimeric mouse, which is a blend of both the normal cells and the cells containing the targeted *Cftr* gene. With the use of this basic technique, mouse models have been generated which have been designed to disrupt the *Cftr* gene, either by using a replacement gene-targeting strategy resulting in the creation of animals with no normal Cftr production (*Cftr* ^{tm1Unc}, *Cftr*^{tm1Cam}, *Cftr*^{tm1Hsc}, *Cftr*^{tm3Bay}) (Snouwaert *et al*., 1992, Ratcliff *et al*., 1993, Rozmahel *et al*., 1996, Hasty *et al*., 1995), or using insertion into the target gene with no loss of genomic material (C*ftr*$^{TgH(neoim)Hgu}$, *Cftr*^{tm1Bay}) (Dorin *et al*., 1992, O´Neal *et al*., 1993). Mouse models designed to reproduce known clinical mutations have been created using either replacement gene targeting (*Cftr*^{tm2Cam}, *Cftr*^{tm1Kth}, *Cftr*^{tm1G551D}) (Colledge *et al*., 1995, Delaney *et al*., 1996,

Zeicher *et al*., 1995)or double homologous recombination procedure ('hit and run') (*Cftr*^{tm2Hgu}, *Cftr*^{tm1Eur}) ((Dickinson *et al*., 2002, van Doorninck *et al*., 1995).

1.3) Phenotypes of mouse models of CF

The phenotype among the CF mouse models is fairly similar with variations which relate to the specific mutation generated, to environmental influences and to independently segregating modifier genes.

1.3.1) Survival of mouse models

The survival rates of the different mouse models vary from <5% in the *Cftr*^{tm1Unc} (Snouwaert *et al*., 1992)**,** *Cftr*^{tm1Cam} (Ratcliff *et a*l., 1993) *Cftr*^{tm1Hsc} (Rozmahel *et al*., 1996) with no wild type Cftr mRNA detectable and the *Cftr*^{tm2Cam} **(**Colledge *et al*., 1995) with 15% of normal expression levels of mutant Cftr mRNA, to 90% survival in the C*ftr*$^{TgH(neoim)Hgu}$ (Dorin *et al*., 1992), which has shown to have 10% of normal levels of wild type Cftr mRNA to normal survival as seen in the *Cftr*^{tm2Hgu} (Dickinson *et al*., 2002) and *Cftr*^{tm1Eur} (van Doorninck *et al*., 1995) mouse models with mutant Cftr mRNA expression at normal levels. The most predominant cause of mortality of the CF mouse models is the severity of intestinal pathology, which appears to be the hallmark of the induced mutations in the murine *Cftr* gene. However, these differences in the rates of survival reflect that severity of intestinal pathology vary as a result of certain mutation specific effects such as residual activity of wild type Cftr mRNA. The low level 10% of wild type Cftr has been proposed to be the explanation for the significantly greater survival rate in the C*ftr*$^{TgH(neoim)Hgu}$ mouse model (Dorin *et al*., 1994b). Because of the targeting strategy used in its generation, exon skipping and aberrant splicing produce normal Cftr mRNA (Kent *et al*., 1996), resulting in a much milder gastrointestinal phenotype than exhibited by the other knockout mouse models. Other factors that have been shown to influence the survival rate of the various mouse models is the environment in particular the diet and the housing conditions. The lifespan of the *Cftr*^{tm1Unc} mouse model has

been prolonged when solid diet was replaced with liquid diet (Kent *et al*., 1996). Delaney *et al*., (1996) have shown that survival of the *Cftr*^{tm1G551D} mouse model was influenced by the sterility of the housing conditions. Transfer of the *Cftr*^{tm1G551D} mice from a specific pathogen-free (SPF) environment to a standard animal facility resulted in increased mortality.

1.3.2) Intestinal disease

In the human subject, the intestinal manifestation of CF in the small intestine is characterized by decreased Cl^- and fluid secretion, which may contribute to the meconium ileus (MI) in approximately 10% of CF newborns and intestinal obstruction and accumulation of mucus in older CF patients (Eggermont *et al*., 1991). The intestinal phenotype appears to be the hallmark of all CF mouse models. With the exception of two of these models, *Cftr*^{tm2Hgu} (Dickinson *et al*., 2002) and *Cftr*^{tm1Eur} (van Doorninck *et al*., 1995), the intestinal pathology is severe. Mucus accumulation in the crypts of Lieberkuhn, goblet cell hypertrophy (*Cftr*^{tm1Unc}) (Eckman *et al*., 1995), hyperplasia (*Cftr*^{tm1Unc}) (Eckman *et al*., 1995), *Cftr*^{tm1Hsc} (Rozmahel *et al*., 1996) and *Cftr*^{tm1Bay} (O´Neal *et al*., 1993), and eosinophilic secretions in the crypts (*Cftr*^{tm1G551D}, *Cftr*^{tm3Bay} , *Cftr*^{tm1Bay} , *Cftr*^{tm1Eur}) (Delaney *et al*., 1996, Hasty *et al*., 1993, O´Neal *et al*., 1993, van Doorninck *et al*., 1995) are some of the intestinal pathology features observed in CF mouse models.

Intestinal mortality and morbidity in CF mice manifests during two time periods before weaning and after. A large fraction of CF mice die shortly after birth from severe intestinal complications (*Cftr*^{tm1Kth}, *Cftr*^{tm1Bay}, *Cftr*^{tm1Hsc}), whereas others die shortly after the weaning period (*Cftr*^{tm1Unc}, *Cftr*^{tm1Cam}). Consumption of solid food has been postulated to be the causative reason for the post weaning high rate of mortality in CF mice, it has been shown that liquid diet prolongs the lifespan of these animals (Eckman *et al*., 1995, Kent *et al*.,1996). Furthermore Grubb *et al*., (1995) found that substitution of the drinking water with an

electrolyte solution containing 6% polyethylene glycol (Colyte), greatly prolonged the life span of the *Cftr*^{tm1Unc} mouse model.

With respect to intestinal physiology, all of the CF mouse models have a very similar physiological phenotype to that of the human intestine which corresponds to defective cAMP-mediated Cl^- conductance. Studies characterising the electrophysiological profile of the intestine in CF mouse models revealed great similarity between the intestinal phenotype of the various mouse models. CF mouse models display a significant decrease (*Cftr*^{tm1G551D}, C*ftr*$^{TgH(neoim)Hgu}$, *Cftr*^{tm1Eur}) (Delaney *et al*., 1996, Smith *et al*., 1995, van Doorninck *et al*., 1995) or complete absence of cAMP-mediated Cl^- secretion across the intestinal epithelium (Clarke *et al*., 1992, Colledge *et al*., 1996, Hasty *et al*., 1995, Ratcliff *et al*., 1993., Zeicher *et al*., 1995). In the *Cftr*^{tm1Unc} mouse model, all intestinal regions from the duodenum to the distal colon exhibited defective cAMP-regulated Cl^- ion transport (Grubb *et al*., 1997). In the C*ftr*$^{TgH(neoim)Hgu}$ mouse the residual function nature of the insertional mutation has been postulated to be the cause of the observed mild instestinal disease phenotype in these mice (Dorin *et al*., 1994b).

In contrast to the two F508del mouse models *Cftr*^{tm1Kth}and *Cftr*^{tm2Cam} the *Cftr*^{tm1Eur} F508 del mouse model does not exhibit increased mortality due to intestinal complications and shows a substantial Cl^- secretory response to an increase in cAMP (van Doorninck *et al*., 1995). *Cftr*^{tm1Kth} and *Cftr*^{tm2Cam} exhibit a reduction in mRNA levels for the mutated Cftr (Zeicher *et al*., 1995, Colledge *et al*., 1996) whereas the *Cftr*^{tm1Eur} mouse model expresses mRNA levels for the mutated CFTR comparable to the levels of the wild type control littermates (French *et al*., 1996). It has been speculated that this high level of mRNA may allow more of the mutant CFTR to be correctly processed allowing more functional protein to reach the plasma membrane.

With respect to Ca^{2+} activated Cl^- secretory responses the intestine of the CF mouse models is similar to that of its human homolog, Like the normal human intestine normal murine

intestine reacts to agonists that increase intracellular Ca^{2+} with a Cl^- secretory response, whereas human CF patients and mice lacking functional CFTR as the *Cftr*^{tm1Unc}/*Cftr*^{tm1Unc} , *Cftr*^{tm3Bay}/*Cftr*^{tm3Bay} (Hasty *et al*., 1993, Grubb *et al*., 1997, Cuthbert *et al*., 1994, Clarke *et al*., 1994) CF mouse models exhibit no Cl^- secretory response to these agents.

1.3.3) Lung disease

The major cause of morbidity and mortality in CF humans is due to pulmonary manifestations of the disease, therefore, the airways of the CF mouse models is of major interest. Unlike the reports of severe gastrointestinal pathology in the CF models there is an observed lack of pulmonary pathology.

For the *Cftr*^{tm1G551D}, *Cftr*^{tm1Unc}, *Cftr*^{tm1Kth} CF mouse models the findings with respect to airway hyperabsorption of Na^+ across the nasal mucosa is consistent with what is seen in CF patients as indicated by a significantly enhanced baseline nasal PD in vivo (Delaney *et al*., 1996, Grubb *et al*., 1994, Wilschanski *et al*., 1996, Zeicher *et al*., 1995). Decreased or even absent cAMP-stimulated Cl^- conductance was observed in all of the CF mouse models tested with the exception of the *Cftr*^{tm1Eur} (van Doorninck *et al*., 1995) and the C*ftr*$^{TgH(neoim)Hgu}$ (Smith *et al*., 1995) models. The approximate 10% of wild type Cftr mRNA in the lung of the C*ftr*$^{TgH(neoim)Hgu}$ mouse model and the normal levels of F508del Cftr in the *Cftr*^{tm1Eur} mouse could explain, according to the authors, the response to the low Cl^- perfusion (van Doorninck *et al*.,1995, Smith *et al*., 1995). In the human airway tissue, stimulated Cl^- secretion is mediated almost equally by the Cftr channel and a molecularly distinct, alternative Ca^{2+} regulated channel in the apical membrane (Boucher *et al*., 1989). The nasal mucosa of *Cftr*^{tm1Unc} and *Cftr*^{tm1Hsc} mice have been reported (Grubb *et al*., 1994, Wilschanski *et al*., 1996) to display a raised Ca^{2+}- mediated Cl^- secretory pathway which could partially alleviate the effects of Cftr dysfunction in these mouse models.

The physiology of the murine distal airways (trachea, bronchi) appears to be different from that of the upper airways (nasal mucosa). Reports from studies on the tracheas of CF mouse models such as the *Cftr*^{tm1UNC} (Grubb 1994a), *Cftr*^{tmG551D} (Delaney *et al*., 1996) *Cftr*^{tm2Cam} (Colledge *et al*., 1995) mice reported no difference in the amiloride-sensitive Isc of the CF tracheas when compared with that of the normal trachea. A reduction in the amiloride-sensitive Isc in the CF murine trachea when compared with the normal was also reported for the *Cftr*^{tm1Cam} (Hyde *et al*., 1993) and C*ftr*$^{TgH(neoim)Hgu}$ (Smith *et al*., 1995) mouse models, the authors suggested that in contrast to humans the loss of Cftr does not result in the upregulation of the epithelial Na^+ channel in the murine trachea. Zeicher *et al*., (1995) reported the presence of little or absent Cftr expression in the tracheas of normal mice and the existence of an alternative non-Cftr Cl^- channel has been proposed. Studies on the *Cftr*^{tm2Cam} (Colledge *et al*., 1995) showed that in CF animals between the age of 18-32 days the forskolin response was present but significantly reduced from that in the normal trachea. A similar pattern was detected in the trachea of the *Cftr*^{tmG551D} mouse model (Delaney *et al*., 1996). In the two knockout CF models (*Cftr*^{tm1UNC}, *Cftr*^{tm1Cam}) the cAMP mediated Cl- secretory response was found to be identical with that of the normal trachea. Cftr mediated Cl^- secretion could not be supported in these animals due to lack of functional Cftr supporting the hypothesis that an alternative Cl^- secretory pathway. A study using the *Cftr*^{tm1UNC} mouse model (Grubb 1994a) revealed that the cAMP- mediated Cl^- response in the murine CF trachea is via an intracellular Ca^{2+}- mediated non-Cftr pathway.

1.3.4) Lung pathology

CF airways are particularly susceptible to chronic bacterial infection and intense inflammation that results in significant tissue damage and eventual failure of lung function. Common bacterial pathogens that infect the CF lung include *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* (PA). PA persists in the airways forming, forming

microcolonies embedded in a mucoid exopolysaccharide called alginate (Pilewski *et al*., 1999). Initial studies using the outbred MF1/129- C*ftr*$^{TgH(neoim)Hgu}$ mouse model no gross lung disease was observed at birth or in those animals born and raised in isolator conditions (Davidson *et al*., 1995). Evaluation of the cytokine levels in bronchoalveolar lavage fluid (BALF) from untreated MF1/129 C*ftr*$^{TgH(neoim)Hgu}$ mice revealed significantly increased levels o the pro-inflammatory cytokine tumour necrosis factor a (TNF-α) in the mutant mice in comparison with non-CF controls (Morrison *et al*., 1997).

One indicator of the chronic inflammatory status of CF patients is the presence in the serum of two S-100 calcium- binding proteins, S100A8 and S100A9 (Barthe *et al*., 1991). S100A8 was identified independently in mouse as a powerful proinflammatory chemotactic factor (Lackmann *et al*., 1992) while both S100 proteins also exhibit antimicrobial activity. The expression of S100A8 mRNA in murine macrophages is inducible by bacterial LPS (Hu *et al*., 1996). Thomas *et al*., (2000) using the *Cftr*^{tm1G551D} CF mouse model demonstrated that intravenous injections of lipopolysaccharide (LPS) into these mutant mice induced S100A8 mRNA in the lung to a greater extent than in wild type litermates. Bone marrow-derived machrophages from the same mice were shown to also exhibit hypersensitivity to LPS, measured by induction of TNF-α, providing evidence that the pathology of CF relates to abnormal regulation of the immune system. McMorran *et al*., (2001) using again *Cftr*^{tm1G551D} mice examined the response of the animals during the early acute phase of infection with PA, between 1 and 3 days after bead inoculation. Concentration of TNF-α was significantly higher than in non-CF littermates 24h after infection, followed by a rapid loss of weight. 48h after infection a decline of inflammatory cytokines was observed, the authors speculated that this decline could predict the ineffective bacterial clearance. In studies using the MF1/129/- C*ftr*$^{TgH(neoim)Hgu}$ mouse model an impaired airway clearance of aerosolised *Staphylococcus aureus* and *Burkholderia cepacia*, in comparison with non-CF animals was demonstrated (Davidson *et al*., 1995). Repeated exposure to these bacteria resulted in formation of

lymphocytic aggregates, mucus retention, bronchiolitis, pneumonia and oedema. The same results were obtained from C*ftr*$^{TgH(neoim)Hgu}$ mice bred to the inbred C57BL/6 genetic background (Cowley *et al*., 1997). Coleman *et al*., (2003) demonstrated that under the proper conditions, transgenic *Cftr*^{tm1Unc} mice are hypersusceptible to *P.aeruginosa* colonisation and infection. When the bacterium was present in drinking water, heterozygous and homozygous CF mice housed in the same cage became chronically colonized in the oropharynx with environmental PA. Elimination of PA from drinking water resulted in clearance in most wild type and CF heterozygous but not homozygous mice.

1.3.5) Pancreatic disease

In human CF patients, pancreatic insufficiency is a prominent manifestation of CFTR dysfunction. The acinar epithelia secrete digestive enzymes, and the CFTR-expressing ductal epithelia secrete a HCO_3-rich liquid that flushes the enzymes into the duodenum. In the human CF patient mucins plug the pancreatic ducts leading to inspissated luminal proteins. Digestive enzymes which are secreted by the acinar epithelia accumulate in the acini due to the blocked ducts, leading to enlarged acini, autolysis and their replacement with fibrotic tissue . In contrast to what is seen in the human CF patient, pancreatic disease is less severe in the CF mouse models. Studies on the F508del and G551D CF mouse models did not report any severe pancreatic pathology (Colledge *et al*., 1996, Delaney *et al*., 1996, van Doorninck *et al*., 1995, Zeicher *et al*., 1995). A more variable pancreatic pathology has been reported for the knockout CF mouse models. Studies using the *Cftr*^{tm1Cam} mouse model reported blockage of some of the pancreatic ducts in approximately 50% of the mice examined, but the pancreatic function was not altered (Ratcliff *et al*., 1993). *Cftr*^{tm1Bay} and *Cftr*^{tm3Bay} exhibited acinar atrophy which progressed as the mice aged (O´Neal *et al*., 1993, Hasty *et al*., 1995). In the *Cftr*^{tm1Unc} knockout mouse model, two of five mice examined exhibited enlarged acini containing eosinophilic material in one or two lobes of the pancreas (Snouwaert *et al*., 1992).

The C*ftr*$^{TgH(neoim)Hgu}$ mouse model exhibits no pancreatic pathology probably due residual amounts of wild-type CFTR (Gray *et al*., 1994).

Electrophysiological experiments using normal pancreatic ductal cells with Ussing chambers (Clarke et al., 1994) and whole cell patch clamp (Gray *et al*., 1994, Winpenny *et al*., 1995) suggested the existence of an alternative fluid secretory pathway which is activated by increases in intracellular calcium ions (Ca^{2+}) consistent with a previous study (Snouwaert *et al*., 1992) which reported low level of *Cftr* expression in the murine pancreas.

1.3.6) Reproductive tissue

Most male human CF patients are infertile because of atresia or obstraction of the vas deferens and distal epididymis (Heaton *et al*., 1990). Snouwaert *et al*., (1992) reported high levels of Cftr mRNA expression in the testes and epididymis of the *Cftr*^{tm1Unc} mouse model, and no pathology of the male reproductive tract has been reported for any of the CF mouse models characterised in literature. It was found that normal tissue exhibited a cAMP-mediated (forskolin) Cl^- secretory response, which was lacking in the CF testes (Leung *et al*., 1996). A larger Cl^- response was elicited by agents that raise intracellular Ca^{2+} such as ionomysin and ATP and this Ca^{2+} mediated Cl^- secretion was found to persist to the same level in the male reproductive tissue from the CF mice. It was speculated by the same author that fertility in the male CF mouse is maintained by the presence of the predominant Ca^{2+} mediated Cl^- secretory pathway in the epididymides and seminal vesicles. Female C*ftr*$^{TgH(neoim)Hgu}$ and *Cftr*^{tm1Kth} mice are fertile, whereas *Cftr*^{tm1Cam} and *Cftr*^{G551D} homozygous mice are mostly infertile. In a study (Leung *et al*., 1996) on primary cultures of murine oviductal epithelium from normal and *Cftr*^{tm1Unc} mice it was reported that like in the male reproductive tissue, ATP-stimulated Ca^{2+} –mediated Cl^- secretory response was significantly higher than the cAMP-mediated Cl^- secretory response which was maintained also in the CF mouse.

1.4) Phenotype Modification

1.4.1) Genetic Background

One of the advantages when working with mice is the availability of inbred strains, according to the committee on Standardised Genetic Nomenclature for mice (1952) a strain is considered to be inbred after at least 20 consecutive generations of brother x sister mating, which can be traced to a single ancestral breeding pair. An inbred strain is genetically uniform (isogenic) and utilisation of these strains in research helps to eliminate the genetic variation.

Along with the environmental conditions such as diet or housing conditions another factor actively influencing the severity of the phenotype and hence the different survival rates of the various CF mouse models is the background strain. However, generation of the various CF mouse models did not put emphasis upon the choice of the background strain used, and initial characterisation of these mouse models did not take into account the role that the background strain may have upon the phenotype. The severity of lung disease in CF mice kept alive on a liquid diet has been shown to be influenced by genetic background (Kent *et al*., 1997). In order to dissect the role of the mutation from the genetic background the CF mouse models are backcrossed with chosen inbred strains and the resultant offspring is congenic with 99.9% inbred background after ten backcrosses. Establishment of congenic CF mutant animals results in the generation of CF mouse models which differ only in the nature of the mutation in *Cftr*. In their study Kent *et al*., (1997) demonstrated that intergrossing of the $Cftr^{tm1Unc}$ mutation the inbred C57BL/6J genetic background (congenic) resulted in lung disease in the absence of detectable infection absent in $Cftr^{tm1Unc}$ CF animals of a mixed genetic background.

1.4.2) Modifier loci

Mouse models present powerful means to identify secondary genetic modifiers (Nadeau 2001). Establishment of different inbred strains with the same *Cftr* mutation (congenic), allowed the comparison between various inbred strains facilitating the search of

independently segregating modifier loci. Rozmahel *et al*., (1996), mapped a modifier gene locus on chromosome 7 (*Cfm1*). On the basis of the human-mouse homology map, Zielenski *et al*., (1999) demonstrated the existence of a genetic locus (CMF1), presumably equivalent to *Cmf1*, that could determine the susceptibility to meconium ileus in CF patients.

Haston *et al*., (2002a), using quantitative trait loci (QTL) mapping on the F2 (B6xBALB/c) of congenic strains in which the *Cftr*^{tm1Unc} mutation (Snouwaert *et al*., 1992) was crossed with C57BL/6J and BALB/c inbred mice, identified putative CF body weight modifiers on chromosomes 13, 7 and 10. Significant linkage of the fibrotic lung phenotype was also detected (Haston *et al*., 2002b) for a region on Chromosome 6.

Scope of this thesis

- ***Background*** The ability to selectively alter the mouse genome has given a great boost to the research of cystic fibrosis (CF) with the generation of mouse models of CF. The availability of inbred i.e. isogenic mouse strains along with the ongoing progress on the mouse genome provided scientists with a useful tool whereby the complexity of inherited genetic disorders such as cystic fibrosis can be analysed. Moreover, the ability to introduce the mutation on to a different inbred genetic background generating congenic animals facilitated the search for modulators of CF. At present there are several mouse models of CF described in the literature with variable degrees of characterisation, in the context of this thesis the two inbred CF strains CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ are being genetically and phenotypically characterised.

  *Generation of CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$.* Dorin *et al*., 1992 described the generation of a mouse model of CF using insertional mutagenesis to disrupt the murine *Cftr* gene in exon 10. For the establishment of the inbred population a pair of mixed genetic background was obtained from the MRC Human Genetics Unit, Edinburgh. Two separate litters were obtained and two animals of each litter became the starting population for the establishment of the two individual inbred C*ftr*$^{TgH(neoim)Hgu}$ lines CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ which were generated by brother x sister mating for now more than 26 generations.

- ***Objectives of the study*** The study as described in this thesis has three major aspects: the phenotypic characterisation of the two inbred CF strains CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$; their genetic characterisation by using microsatellite markers; and the estimation of their genetic relationship with the three wild type inbred strains

BALB/c, C57BL/6J and DBA/2J, which were used for the generation of congenic C*ftr*$^{TgH(neoim)Hgu}$ animals.

Chapter 2 describes the use of an alternative PCR based protocol whereby identification of the insertional mutation in the recipient inbred strains (during generation of congenic animals) was established using *Cftr* intragenic microsatellite markers linked with the disrupted locus (*Cftr* exon 10). The feasibility of this type of characterisation is discussed along with the finding of vector excision revealing the instability of the construct used to generate the mutant animals.

Chapter 3 deals with the phenotypic characterisation of the two inbred CF strains CF1-C*ftr*$^{TgH(neoim)Hgu}$ and CF3- C*ftr*$^{TgH(neoim)Hgu}$. This characterisation comprised of mRNA analysis along with Cftr protein detection using western blot with the R3195 CFTR antibody in the intestine followed by immunocytochemical analysis both in the nose and the intestine. The electrophysiological defect of cystic fibrosis in the two inbred CF strains CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ was assessed using short circuit measurements (SCC) both in the nose and the intestine and was compared along with wild type control animals and F508del homozygous C*ftr*^{tm1Eur} mutants.

Chapter 4 describes the genetic characterisation of the two inbred CF strains CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and the three inbred wild type strains C57BL/6J, DBA/2J and BALB/c by using microsatellite markers. The aim of this study was to identify the strain distribution patern between the two CF strains and the three inbred strains before analysing the introgressed C*ftr*$^{TgH(neoim)Hgu}$ mutation in the three inbred backgrounds.

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Chapter 2

Instability of the insertional mutation in *Cftr*$^{TgH(neoim)Hgu}$ cystic fibrosis mouse model

Abstract

Two inbred *Cftr*$^{TgH(neoim)Hgu}$ mouse lines CF/1 and CF/3 were generated from the original *Cftr*$^{TgH(neoim)Hgu}$ cystic fibrosis mouse model by brother x sister mating. Thereafter the insertional mutation was introgressed from CF/3 into three inbred backgrounds (C57BL/6, BALB/c, DBA/2J). Transmission of the insertional mutation during backcrossing was monitored by direct probing the insertion via Southern RFLP and indirectly by informative marker haplotype using intragenic *Cftr* microsatellites. This method facilitated the identification of unexpected events such as recombination in the *Cftr* gene during congenic breeding between the wild type and the mutant allele as well as precise vector excision from the disrupted *Cftr* locus. This reversion to wild type status took place without any loss of sequence revealing the instability of insertional mutations during the production of congenic animals.

2.1) Introduction

Cystic fibrosis (CF) is a common and fatal recessive disease, which is caused by dysfunction of a chloride channel, termed the CF transmembrane conductance regulator (CFTR). Since the isolation of the murine homologue of the human *CFTR* gene on Chromosome 6 (Tata *et al*., 1991) several mouse models have been created. These fall broadly into two different categories; those designed to mimic clinical human mutations such as the F508del (Colledge *et al*., 1995; van Doorninck *et al*., 1995; Zeiher *et al*., 1995)**,** G551D (Delany *et al*., 1996) and G480C (Dickinson *et al*., 2002), and those with a disrupted *Cft*r gene resulting in either no or reduced production of CFTR. Although most mouse models share phenotypic characteristics, particularly, the most CF-like severe pathology is observed in the gastrointestinal tract, important variations in phenotype have been observed which may relate to the specific mutation and the genetic background of the targeted strain. Studies using *Cftr* knockout mice demonstrated differential severity of airway (Kent *et al*., 1997) and intestinal (Rozmahel *et al*., 1996) disease. Candidate modulators for growth, airway and intestinal disease have been mapped to loci on chromosomes 1, 6, 7, 10 and 13 (Haston *et al*., 2002a) ; 1, 2, 10 and 17 (Haston *et al*., 2002b); 3 and 5 (Haston *et al*., 2002c), respectively.

Dorin *et al*. (1992) established a CF mutant mouse C*ftr*$^{TgH(neoim)Hgu}$, using an insertional gene targeting vector to disrupt exon 10 of the *Cftr* gene in 129P2 embryonic stem cells. This targeted mutation was made by insertional mutagenesis using a fragment of DNA containing intron 9 and part of exon 10 (Figure 1). The mutation is slightly "leaky", in that low levels of wild type *Cftr* mRNA are produced as a result of exon skipping and aberrant splicing (Dorin *et al*. 1994), but these mutant mice nevertheless displayed the electrophysiological defect in the gastrointestinal and respiratory tract which is characteristic of CF (Smith *et al*. 1995). We have generated two different inbred lines named CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ using brother-sister mating for more that 26 generations. In order to test

whether the genetic background of the C*ftr*$^{TgH(neoim)Hgu}$ mouse influences the development of the phenotype, we introgressed the mutation from the CF/3- C*ftr*$^{TgH(neoim)Hgu}$ into three different inbred strains (C57BL/6, BALB/c, DBA/2J) generating B6.129P2(CF/3)-C*ftr*$^{TgH(neoim)Hgu}$, C.129P2(CF/3)- C*ftr*$^{TgH(neoim)Hgu}$, D2.129P2(CF/3)- C*ftr*$^{TgH(neoim)Hgu}$ congenic mice. During backcrossing the targeted mutation was determined by Southern RFLP analysis of *Xba*I/*Sal*I genomic digests with probe 1.2H (Figure 1) as outlined in the original report (Dorin *et al*., 1992). Here we describe an alternative genotyping technique utilising informative *Cftr* intragenic microsatellite markers in order to follow germline transmission of the mutated *Cftr* locus in the three inbred backgrounds. The four markers spanning 101kb of the *Cftr* gene allowed straight forward differentiation between the two inbred CF lines and the three inbred wild type strains by microsatellite haplotype. Southern and microsatellite mutation genotypes were confirmed in 55 of 57 typed mice two of which harboured a recombination in the *Cftr* gene. In two cases, however, the insertion mutation status deduced from Southern hybridisation and microsatellite genotypes did not match. Further mapping and sequencing revealed that the 7.3kb insertion vector had been excised from the *Cftr* locus. This spontaneous reversion to wild type sheds serious doubts for the stability of insertion mutations in heterozygous mice.

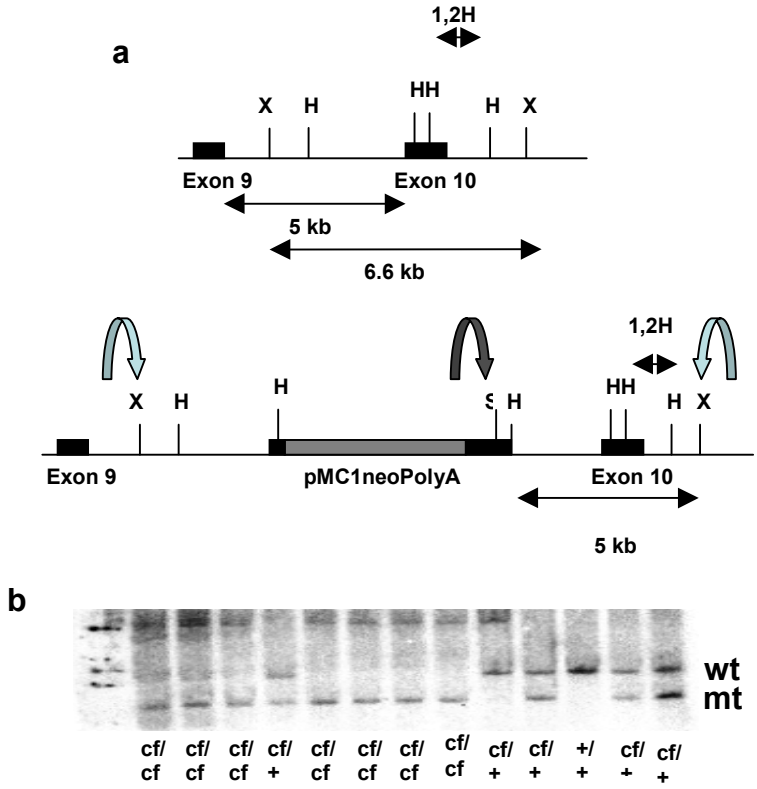


Figure 1. a) Insertional disruption of the murine *Cftr* gene and predicted gene structure as described by Dorin *et al*., (1992). Abbreviations: S, *Sal*I; H, *Hind*III; X, *Xba*I. **b)** Genotype analysis of heterozygous cf/+ matings. The probe 1.2H isused in order to identify via Southern hybridisation the congenic mice which carry the insertion. DNAs were digested with *Xba*I+*Sal*I and probed with 1.2H. The upper hybridising fragment of 6.6Kb represents the wild type allele, the lower 5Kb fragment is diagnostic for the insertional mutation

2.2) Materials and Methods

2.2.1) Experimental animals.

All experiments were approved by the local Institutional Animal Care and Research Advisory Committee as well as by the local government. C*ftr*$^{TgH(neoim)Hgu}$ mice were bred under specified pathogen-free conditions in the isolator unit of the Central Laboratory Animal Facility of the Hannover Medical School. Mice were kept in a flexible film isolator. The temperature within the isolator was maintained at 20-24ºC with 40-50% relative humidity. Animals were fed an irradiated (50 kGy) standard chow (Altromin 1314) and autoclaved water (134ºC for 50min) ad libitum.

2.2.2) Generation of inbred Cftr$^{TgH(neoim)Hgu}$ mutant mice.

For the establishment of the inbred CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ population, one pair with divergent genetic background (generation F4) of one homozygous male and one homozygous female was obtained from the MRC Human Genetics Unit, Edinburgh. Two separate litters were obtained and two animals of each litter became the starting population for the establishment of the two individual inbred C*ftr*$^{TgH(neoim)Hgu}$ lines CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ which were generated by brother-sister mating for now more than 26 generations.

2.2.3) Generation of congenic Cftr$^{TgH(neoim)Hgu}$ mutant mice.

CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mice served as donors for the development of the three congenic strains C57BL/6, BALB/c and DBA/2J, with selection for C*ftr*$^{TgH(neoim)Hgu}$ for 10 generations. Genotyping of the insertion mutation was conducted by Southern analysis of *Xba*I/*Sal*I restricted genomic DNA from spleen (Dorin *et al*., 1992).

2.2.4) DNA purification.

High molecular weight DNA was isolated from 0.15g spleen tissue, either fresh or thawed on ice after storage at -20ºC based on the protocol by Gross-Bellard *et al*., (1973).

2.2.5) Southern blot genotyping.

Heterozygous and homozygous C*ftr*$^{TgH(neoim)Hgu}$ animals were identified in each backcross generation via Southern Blot Hybridization of *Xba*I/*Sal*I genomic digests, using the 1.2H probe located in the *Cftr* intron 10, after double digestion with *Xba*I-*Sal*I (Figure 1). There are no *Sal*I sites in this region of the *Cftr* gene, but the targeting vector pIV3.5H carries a unique *Sal*I site immediately 3´ to the *neo* gene. Animals carrying the mutation were identified by the novel 5kb *Xba*I-*Sal*I fragment hybridizing to 1.2H.

2.2.6) Microsatellite selection.

The sequence available in the Genome Database (AF162137) was used for manual selection of dinucleotide repeat units spanning the murine *Cftr* gene. Five microsatellite markers were identified in *Cftr* intron 1 (D6NC3), intron 2 (D6NC4), intron 8 (D6NC2), intron 10 (D6NC1) and intron 18 (D6NC5). Flanking primers designed with the oligonucleotide designing program Primer 3 (www-genome.wi.mit.edu/cgi-bin/primer3.cgi/primer3_www.cgi) are listed in Table 1.

Primer name	Primer sequence 5´-3´
D6NC1-A	BIOTIN-TGC TTG AGC TAT CCA TTC TGA
D6NC1-B	TAC CCA ATG TTG CCA TCT GA
D6NC2-A	BIOTIN-TTG GAA GTG AGG ATT GCC TT
D6NC2-B	TGC CTC AGT CTC ATA TTA TTG C
D6NC3-A	BIOTIN-TCT CAG CCT GTC TTC CTC TCA
D6NC3-B	TCC TCC CAA AAC AGC TTC AC
D6NC4-A	BIOTIN-GAG TTG GAG AGG CTG TTT GG
D6NC4-B	TGT GCC AGG ACA CTG TGA CT
D6NC5-A	BIOTIN-TTC AAA TGA CCA AAA TCC CC
D6NC5-B	TGG CAA ATT TTC AAC AAC AAA

Table 1. Primer sequences used for the amplification of the intragenic *Cftr* microsatellites. The forward primer is 5´biotinylated.

2.2.7) Genotyping of microsatellites.

Microsatellite markers were genotyped in 96 well plates purchased from Greiner, Frickenhausen, pre-coated with 50ng DNA per well in a Hybaid Thermocycler (Hybaid, Teddington) with a heated lid. One of the two primers per microsatellite was 5´-terminal biotinylated. PCR was performed in a total volume of 30µl, without oil overlay, using InViTaq polymerase (InViTek, Berlin). After PCR an 8µl aliquot was transferred to a multiwell plate and allowed to dry overnight at 37ºC, dissolved in 10µl loading buffer (0.2% w/v xylenecyanol and bromphenolblue in formamide) and denatured for 5min at 95ºC. The PCR products were separated by direct blotting electrophoresis (GATC 1500, MWG Biotech, Ebersberg, Germany) on a denaturing acrylamide gel (4% acrylamide/N,N'-methylenebisacrylamide 29:1 containing 6M urea in 0.9M Tris-0.9M boric acid-0.02M EDTA buffer) and simultaneously transferred to a Hybond N+membrane (Amersham). Signals were visualised by blocking the membrane in 1.5%(w/v) of blocking reagent in Buffer 1 (100mM Tris-HCl, 150mM NaCl, pH 7.5), followed by incubation in diluted solution of anti-biotin alkaline phosphatase conjugate in Buffer 1. The membrane was further washed three times with 1% Triton X-100 in Buffer 1 and equilibrated for 15min in assay buffer (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5). The membrane was covered for 5min with reaction buffer containing 10%(v/v) Sapphire II (Tropix) and 60µl CDPstar (Tropix)in 50ml assay buffer, followed by rinsing with a solution containing 1% v/v Sapphire II and 6µl CDPstar in 50ml assay buffer. Signals were exposed to Kodak XA-R films and the exposition time varied from 10min to 45min. Evaluation of results was performed as described by Mekus *et al*., (1995).

2.2.8) Long-range PCR.

150ng of DNA template was each amplified in 12 different premixes using the Failsafe ™ PCR System (EPICENTRE Technologies, WI USA). PCR products were amplified using

primer described in Table 2 separated by 1% agarose gel electrophoresis and visualised under UV illumination, the optimal reaction mixture was thereafter chosen for further amplifications.

Primer name	Primer sequence 5´-3´	Expected size	Allele
Cftr-5012	**CCT TCC ATG TAC CCC TCC TCA CTT CCC GGC ATA ATC CAA GAA AAT TG**	**5012bp**	**Wild type**
Cftr-5198	**TGT GGG AAA TCC TGT GCT GAA A CTT CCG GCT CGT ATG TTG TGT T**	**5198bp**	**mutant**
Cftr-3736	**CAC ACA ACA TAC GAG CCG GAA G TTT ATT GCC GAT CCC CTC AGA A**	**3736bp**	**mutant**
Cftr-3473	**CTC GTG CTT TAC GGT ATC GCC TGC TGT AGT TGG CAA GCT TTG A**	**3473bp**	**mutant**

Table 2. Primer sequences used for the amplification of the long range products.

2.2.9) Neo PCR.

Cftr intron 9-pIV3.5H vector and *neo* -*Cftr* intron 9 spanning primers**,** (Table 3) were amplified using PCR of 50ng DNA template in a total volume of 30µl with InViTaq polymerase (InViTek, Berlin) in 96 well plates. Full-length and *Sal*I restricted PCR products were separated by 2.5% agarose gel electrophoresis.

Primer name	Primer sequence 5´-3´	Expected size
CFneo 1-A	**CGT TGG CTA CCC GTG ATA TT**	**332bp**
CFneo 1-B	**CTT CCA CAA GGC TTC CTG AG**	
CFneo2-A	**CCT GAT GTT GAT TTT GGG AGA**	**253bp**
CFneo2-B	**ATT AAT GCA GCT GGC ACG AC**	

Table 3. Primer sequences used for the amplification of the *Cftr* intron 9-pMC1 vector plasmid sequence (CFneo2) and the neomysin- *Cftr* intron 9 (CFneo1) products.

2.2.10)Excision scanning by primer walking.

Based on the Genome Database *Cftr* sequence (AF162137) 15 overlapping pairs (Table 4) of primers spanning the entire region from exon 9 to intron 10 of the murine *Cftr* gene were designed, using the Primer 3 oligo design program (www-genome.wi.mit.edu/cgi-bin/primer3.cgi/primer3_www.cgi). PCR reactions were performed on DNA with inconsistent Southern and microsatellite insertional mutation genotypes and controls in 96 well plates precoated with 50ng of DNA template using InViTaq polymerase (InViTek, Berlin). Full length products were separated on 2.5% agarose gels and visualised under UV illumination.

Primer name	Location	Sequence 5′-3′	Product Size
NCEx9I9-A	197720- 197742	TTT GGG GAA TTA CTG GAG AAA G	419bp
NCEx9I9-B	198138- 198117	AGC TCG CTG ATA GGT TAT CCA	
NC10-A	198002-198023	CCC CTC CTC ACT TCC ATT AAA	400bp
NC10-B	198402-198381	TTT AAG GCT CAG GGC TAA TTG	
NC11-A	198376- 198396	TTC CAC AAT TAG CCC TGA GC	649bp
NC11-B	199024- 199001	TGA AGG AAA TCA TTA CTG AAG CA	
NC12-A	199001- 199024	TGC TTC AGT AAT GAT TTC CTT CA	550bp
NC12-B	199551- 199531	TAT GGA TCC CCA CAG CAA GT	
NC13-A	199394- 199414	CTC AGG GAT TGT CAC GGT TT	563bp
NC13-B	199966- 199946	GCT TTG ATC TCT GGG AGC AC	
NC14-A	199741- 199763	GAT CAC AGG AGC CTA GCA TAG A	550bp
NC14-B	200290- 200268	TTC ACT TTA CAT CCT GGC TTC A	
NC15-A	200122- 200142	ACT GGG AGA GGA TGC AAA AA	575bp
NC15-B	200696- 200676	CCC AGT GTG AGA AGA TGC AC	
NC16-A	200572- 200592	TGC TCC CAG AAA TCT TCA CC	582bp
NC16-B	201153- 201133	AGT TGT CAG AAG GGA ACC CA	
NC17-A	201134- 201154	TGG GTT CCC TTC TGA CAA CT	582bp
NC17-B	201715- 201695	TTA GGT CCC CGT GCT TAC AC	
NC19-A	201739- 201759	TAG GTG GAT CCA TAA CCC CA	480bp
NC19-B	202219- 202199	GGA CAG AGA AGC AGG AGT GG	
NC20-A	202199- 202219	CCA CTC CTG CTT CTC TGT CC	487bp
NC20-B	202686- 202666	AAA GAA GAG CGA GCC CCT AC	
NCI9Ex10-A	202593- 202612	CCA TAG CCC AAG AGC TTT CA	413bp
NCI9Ex10-B	203007-202987	GTA CCC GGC ATA ATC CAA GA	
NCEx10I10-A	202986-203006	TTC TTG GAT TAT GCC GGG TA	403bp
NCEx10I10-B	203387-203367	TTT CCA GTT GGG GGT ACA CT	
NC21-A	203296- 203316	GGG CTT CAA GGC CTA ATT CT	479bp
NC21-B	203775- 203755	ATG TGA TCC AGA CTG GCC TA	
NC22-A	203654- 203674	ATG CAT GGG GTG TGG TAC TT	625bp
NC22-B	204277- 204255	TCC AAT GAT CTA CCT GTG TCC A	

Table 4. Primer sequences used for primer walking spanning the entire region from *Cftr* exon 9 to *Cftr* intron 10. Location based on the Genome Database *Cftr* sequence (AF 162137)

2.2.11)Sequencing.

Following PCR amplification the chosen PCR products were sequenced by Qiagen GmbH

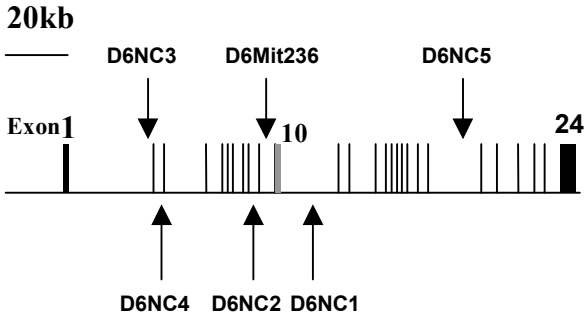
2.3) Results

From the original C*ftr*$^{TgH(neoim)Hgu}$ mutant mouse generated using insertional mutagenesis in the *Cftr* exon 10 (Dorin et al 1992) we have established two inbred CF lines CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ by strict brother sister mating. We have generated three inbred congenic strains by backcrossing the targeted mutation to three different inbred backgrounds C57BL/6, DBA/2J and BALB/c. To observe germline transmission of the mutation after each backcross and after the first incross to develop homozygous congenic strains, mice were genotyped using Southern Blot Hybridisation to indicate the transmission of the insertional vector pIV3.5H (Figure 1). Since Southern analysis is cumbersome and time consuming, we devised an alternative protocol for genotyping, whereby animals are differentiated at the *Cftr* locus by intragenic microsatellite genotypes tightly linked with the intron 9 and exon 10 of *Cftr* chosen for insertion mutagenesis in the C*ftr*$^{TgH(neoim)Hgu}$ mouse mutant.

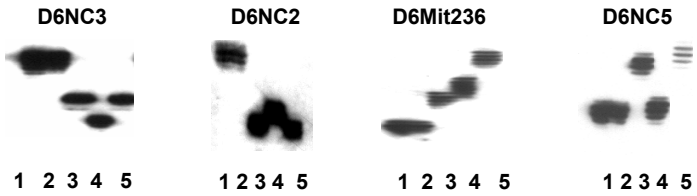
2.3.1) Allele distribution between the strains. Consistent genotyping.

Four of the six tested *Cftr* intragenic microsatellite markers (D6NC3, D6NC2, D6Mit236 and D6NC5) allowed the discrimination of the three inbred strains (C57BL/6, BALB/c, DBA/2J) from the two inbred CF lines CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ (Figure 2). The two inbred CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ lines shared the same marker alleles in all four informative microsatellites being distinct from the three inbred strains. (Figure 2). Hence, a mouse homozygous for the disrupted locus can be identified by the genotype: D6NC3:20/20, D6NC2:20/20, D6Mit236: 20/20, D6NC5: 20/20. Accordingly, the representative genotypes of a a) wild type BALB/c animal will be (D6NC3: 16/16, D6NC2: 13/13, D6Mit236: 31/31, D6NC5: 30/30); b) wild type C57BL/6 (D6NC3: 14/14, D6NC2: 15/15, D6Mit236: 35/35, D6NC5: 21/21); c) wild type DBA/2J(D6NC3: 16/16, D6NC2: 13/13, D6Mit236: 44/44, D6NC5: 32/32).

a



b



c

Mouse strain	D6NC3	D6NC2	D6Mit236	D6NC5
CF/1	20/20	20/20	20/20	20/20
CF/3	20/20	20/20	20/20	20/20
BALB/c	16/16	13/13	31/31	30/30
C57BL/6	14/14	15/15	35/35	21/21
DBA/2J	16/16	13/13	44/44	32/32

Figure 2. a) Localisation of the intragenic polymorphisms on the physiscal map of the Murine *Cftr* gene. **b)** Analysis of the four informative intragenic microsatellites by direct blotting electrophoresis (1=CF/1, 2=CF/3, 3=BALB/c, 4=C57BL/6, 5=DBA/2J). **c)** Microsatellite alleles were ascertained by arbitary repeat units. The alleles for all four microsatellites which are representative of the two CF inbred lines (CF/1 and CF/3)and hence directly linked with the disease causing allele carrying the insertional vector pIV3.5H in *Cftr* exon 10 have been given the number 20.

In order to determine whether germline transmission of the mutation can be accurately assessed via the haplotype of the informative intragenic microsatellites linked to the disrupted *Cftr* locus, we tested all three congenic strains. Fifty-seven animals (C.129P2(CF/3)-C*ftr*$^{TgH(neoim)Hgu}$ n=31, B6.129P2(CF/3)- C*ftr*$^{TgH(neoim)Hgu}$ n= 9 and D2.129P2(CF/3)-

C*ftr*$^{TgH(neoim)Hgu}$ n=17) were compared in the 1.2H probe restriction *Xba*I/*Sal*I RFLP and marker genotypes of the three informative intragenic *Cftr* microsatellites (D6NC3 intron1, D6Mit236 intron 9, D6NC5 intron 18) equally distributed along the *Cftr* gene. Southern RFLP and microsatellite marker genotypes were authenticated for 55 mice. Absence and presence of the insertional mutation in intron 9/exon 10 in homozygous or heterozygous mice could be clearly deduced from the microsatellite genotypes (Figure 2- Table 5).

	1,2H	D6NC3	D6Mit236	D6NC5	
BALB/c					
	n/n	16/16	31/31	30/30	**Homozygote wt**
	cf/n	16/20	31/20	30/20	**Heterozygote mt**
	cf/cf	20/20	20/20	20/20	**Homozygote mt**
C57BL/6					
	n/n	14/14	35/35	21/21	**Homozygote wt**
	cf/n	20/14	20/35	20/31	**Heterozygote mt**
	cf/cf	20/20	20/20	20/20	**Homozygote mt**
DBA/2J					
	n/n	16/16	44/44	32/32	**Homozygote wt**
	cf/n	20/16	20/44	20/32	**Heterozygote mt**
	cf/cf	20/20	20/20	20/20	**Homozygote mt**

Table 5. Expected Southern and microsatellite genotypes for animals backcrossed to the three inbred backgrounds.

2.3.2) Recombination.

Two genotypes (20/16, 20/31 20/20) mouse A and (16/16, 31/31, 20/20) mouse B (Table 6) differed from the expected genotypes in the intragenic microsatellite D6NC5 in intron 18, which is indicative for a recombination between CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and BALB/c chromosomes 3´of the exon 10 of *Cftr*. Refined mapping with the three microsatellites D6Mit50, D6NC3, D6Mit236 upstream of intron 18 and with the two microsatellites D6Mit167 and D6Mit88 downstream of intron 18, revealed that recombinations had occurred within the 100kb upstream and 1.5Mb downstream of intron 18 in one chromosome in mouse A and in both chromosomes in mouse B.

mouse	1,2H	D6NC3	D6Mit236	D6NC5
A	cf/n	cf/n (20/16)	cf/n (20/31)	cf/cf (20/20)
B	n/n	n/n (16/16)	n/n (31/31)	cf/cf (20/20)

Table 6. Genotypes of the animals with recombination 3´of the *Cftr* gene

2.3.3) Excision of the pIV3.5H vector.

In two out of the 57 investigated animals the mutant genotypes as defined by Southern and microsatellite genotypes were discordant (Table 7).

mouse	1.2H	D6NC3	D6Mit236	D6NC5
C	cf/n	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)
D	n/n	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)

Table 7. Genotypes of the animals with an excised vector. Mouse C in one chromosome, Mouse D in both chromosomes.

In detail, mouse C was classified heterozygous CF by Southern and homozygous CF by the microsatellites and mouse D homozygous wild type by Southern and homozygous CF by the microsatellites. Genotyping via Southern Blot Hybridization indicates and depends upon the existence or absence of the insertional vector pIV3.5H designed to disrupt the *Cftr* gene in exon 10. Therefore, as a first step we tried to verify the presence or absence of the pIV3.5H vector with a straight forward PCR assay that scans the ends of the heterologous vector sequence (Figure 3).

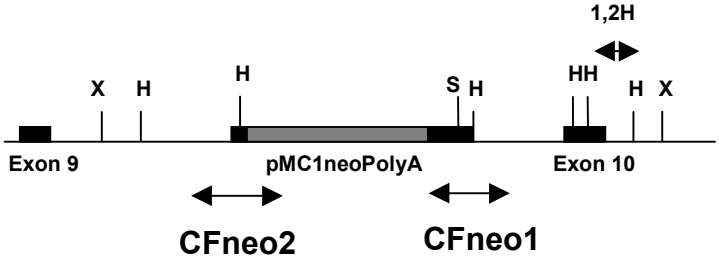


Figure 3. Map position of the CFneo1 and CFneo2 products

One PCR product scans the junctions between intron 9 and the inserted plasmid sequence, the other PCR product the junction between the *neo* gene and the endogenous intron 9 encompassing the unique *Sal*I site. The results of the PCR assays were consistent with the Southern data i.e. for mouse C both insert specific products were present indicating an intact vector on at least one chromosome, whereas for mouse D both products were absent. This data strongly suggests that the pIV3.5H insertion vector had been excised from the CF/3-C*ftr*$^{TgH(neoim)Hgu}$ *Cftr* locus at least in mouse D.

In order to corroborate this suspicion that the vector had been excised in both the heterozygous and homozygous state a long range PCR protocol was established that encompasses the targeted region in intron 9 and exon 10 for both wild type and mutant chromosomes. Four sets of primers were designed (Table 3), one product of 5012bp corresponding to wild type *Cftr* allele and three primer sets to the mutant allele with the inserted sequence (Figure 4).

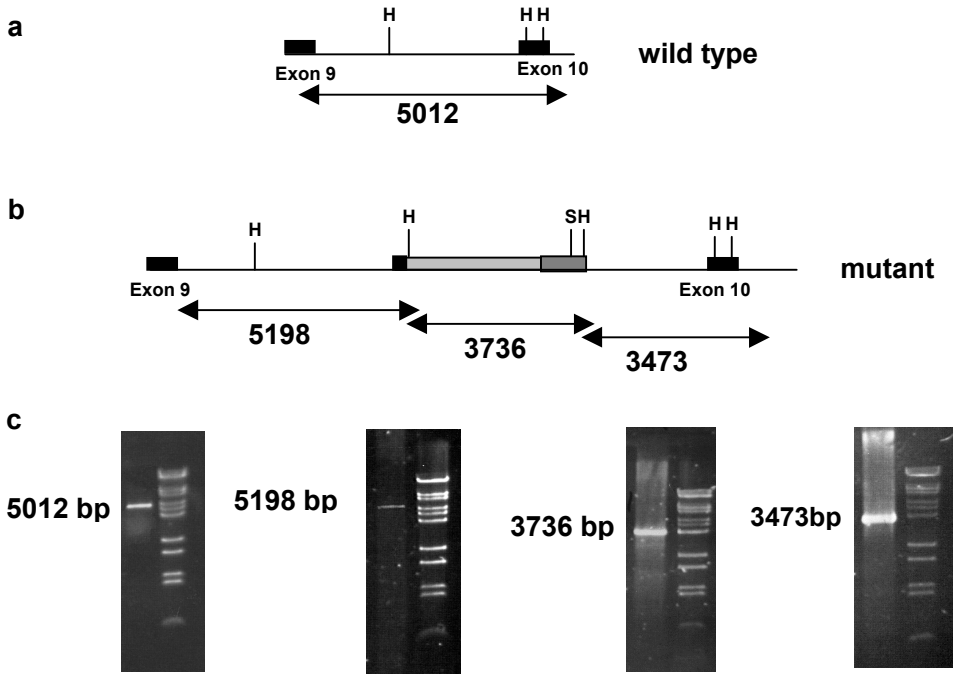


Figure 4. a) Map position of the long range PCR product corresponding to the 5012bp wild type sequence. **b)** Map position of the three long range products corresponding to the mutant allele. **c)** Representative agarose gel (1%) indicating the expected PCR products for all four primer sets.

Mouse D was positive only for the 5012bp product confirming the absence of the pIV3.5H vector on both chromosomes, whereas mouse C was positive for all four products indicative of a CF heterozygous mouse. In mouse D the inserted vector had been excised from both chromosomes, and in mouse C in one chromosome.

2.3.4) Primer Walking.

The sequence integrity of the complete homologous targeted region was checked by primer walking. Fifteen sets of primers were designed from *Cftr* exon 9 to intron 10 (Table 4, Figure 5), and all products of the mutant mice were compared against the BALB/c wild type control. PCR products suspicious for differential migration behaviour on 2.5% agarose compared to those obtained from the wild type BALB/c DNA were sequenced. For all five selected PCR products including NC13 which corresponds to the area in *Cftr* intron 9 where the vector was introduced via homologous recombination (Dorin *et al*., 1992) the sequence was found to be 100% wild type (BALB/c) with small sequence alterations when compared to the AF162137 database C57BL/6 derived sequence. Since the same substitutions were observed for the wild type control they likely represent SNPs between the inbred strains BALB/c, 129P2 and the C57BL/6. In summary, since sequencing by primer walking revealed neither any loss of wild type *Cftr* sequence nor retention of vector sequence, we conclude that in the two mice the pIV3.5H insertion vector had been excised by the base.

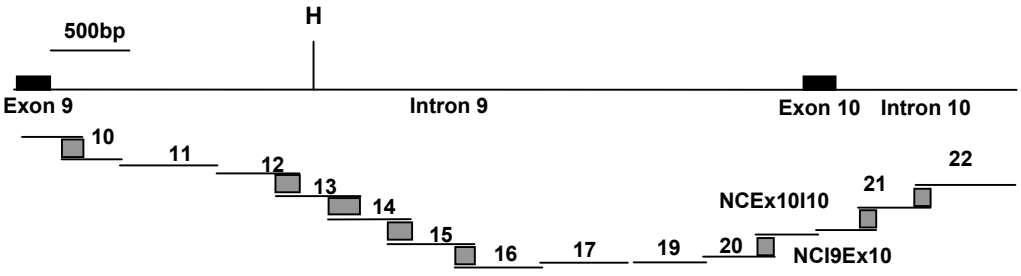


Figure 5. Primer walking. Straight lines represent the fragment amplified by each primer set, overlapping sequences are represented by boxes.

2.4) Discussion

Genetic analysis of complex human diseases such as cystic fibrosis has been successfully supported by the use of various mouse models. In order to dissect the role of the different induced mutations to the murine *Cftr* gene used from the genetic background, the genomic section carrying the mutation is transferred by repeated backcross cycles to another defined inbred background (introgressing), creating congenic strains. We have generated three congenic C*ftr*$^{TgH(neoim)Hgu}$ strains by crossing the mutant animals to the three inbred backgrounds BALB/c, C57BL/6, DBA/2J. In each generation germline transmission of the disrupted *Cftr* locus was monitored using Southern Blot Hybridisation (Dorin *et al*., 1992). In order to observe germline transmission of the disrupted *Cftr* locus we have established an alternative 'high-throughput' genotyping protocol using *Cftr* intragenic microsatellites, which enabled us to identify animals carrying the insertional mutation based upon the different haplotypic backgrounds of the three inbred strains and the mutant CF/3- C*ftr*$^{TgH(neoim)Hgu}$ inbred line at the *Cftr* locus.

The present study is to the best of our knowledge, the first deliberate search for polymorphic intragenic *Cftr* markers for the establishment of *Cftr* haplotypic backgrounds of wild type inbred mouse strains. It has been shown that some of the more common polymorphisms in the human *CFTR* gene have consequences at the functional level. The presence of an allele at a particular locus can determine the proportion of transcripts from which functional CFTR protein can be translated affecting CFTR maturation and the net chloride transport activity of CFTR-expressing cells (Cuppens *et al*., 1998). Although it remains to be proven whether intragenic changes can substantiate for phenotypic variability in disease expression among mice with different *Cftr* background carrying the same mutation, it can not be excluded that they may have a potential effect on the severity of the CF phenotype by several mechanisms.

In our study the determination of the *Cftr* haplotypic backgroung provided a useful tool for the identification of mutant animals. Using this protocol we have successfully verified the

genotype of 55 out of 57 animals bred to the three inbred backgrounds, previously genotyped by Southern blot hybridisation using the 1.2H probe.

2.4.1) Recombination.

A result indicative for a recombination between CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and BALB/c chromosomes 3´of the exon 10 of *Cftr* was observed in two individual cases (mouse A and mouse B). Refined mapping revealed that recombination had occurred within the 100 kb upstream and 1.5Mb downstream of intron 18 in one chromosome in mouse A and in both chromosomes in mouse B. Based on studies done on the human *CFTR* gene recombination breakpoints have been identified between intron 10 and intron 18 and between intron 14a and exon 18 (Morral *et al*., 1996; Dörk *et al*., 1992). The recombination frequency observed in our study is in agreement with the human *CFTR,* with recombination being much more common in the second half of human *CFTR* gene.

2.4.2) Excision.

In two separate cases (mouse C and mouse D) the Southern insertional mutation genotype could not be verified with the three intragenic microsatellites. A heterozygous mouse C and a homozygous wild type mouse D, as indicated via Southern blot hybridisation were homozygous for the intragenic microsatellite genotype linked to the disrupted *Cftr* locus (CF/3- C*ftr*$^{TgH(neoim)Hgu}$ background). Further investigation on these two mice (see Results section) revealed that the outcome of both genotyping methods was correct, supporting the hypothesis of the event of pIV3.5H insertional vector being excised from the mutated *Cftr* locus, on both chromosomes in mouse D and in one chromosome in mouse C. Primer walking revealed that the 7.3kb vector has been excised precisely from the mutated *Cftr* locus without causing any sequence alteration in the *Cftr* gene. Both mouse C and mouse D are the offspring of animals heterozygous for the mutation. The mechanism responsible for this excision repair

event must be independent from the mismatch repair (MMR) and nucleotide exchange repair (NER) pathways, since the size of the vector overexceeds the maximum of mismatched nucleotides they can efficiently repair (Corrette-Bennett *et al*., 2001; Fang *et al*., 2003; McCulloch *et al*., 2003). The mechanism involved in the excision of the vector and the subsequent restoration of the mutated *Cftr* locus to wildtype can not be gene conversion as seen in other organisms (Kearney *et al*., 2001; Clikeman *et al*., 2001), because the genetic background is conserved. If the mechanism involved large loop repair by incorporating the vector in a heteroduplex there must be a novel mechanism, which is independent of gene conversion-restoration events.

O type sequence insertion vectors (Dickinson *et al*., 1993) such as the pIV3.5H, contain an uninterrupted stretch of target- homology with exonic sequence that results in duplication of a large stretch of sequence flanking the heterologous sequence of the plasmid resembling transposable elements, flanked by large direct repeats. Reports (Scott *et al*., 1996) on precise excision events of transposable elements without leaving a footprint involve an alternative mechanism of repair rather than gene conversion which is dependent on length of the repeat flanking the element. It is therefore highly likely that a similar mechanism is responsible for the precise excision of the pIV3.5H insertion vector.

This is the first report where an O type vector used in order to generate insertion mutagenesis in the mouse, has been excised. Such events probably remained unnoticed because most of the methods used in order to identify animals which carry the targeted locus base their detection almost exclusively on the presence or absence of the inserted sequence, without taking into consideration the genetic background of the mouse strain adjacent to the insertion, therefore an excision event would not be easily identified. Unlike Southern hybridisation the genotyping protocol that we propose in this study does not indicate the presence of the insertion vector directly based on the presence of its sequence in the disrupted locus, but manages to discriminate insertional mutant animals from the haplotypes associated with the

disrupted locus in the *Cftr* gene. In our study the haplotypes obtained from the three informative intragenic *Cftr* microsatellites were differential to the haplotypes associated with the insertional mutant mouse, allowing identification of excision events, or any other alteration in the *Cftr* locus including recombination.

2.5) Conclusion

Microsatellite markers spanning the mouse genome have been used for the enhancement of congenic breeding, reducing the time to 18-24 months (speed congenics) from an initial 2.5 -3 year period (Wakeland *et al*., 1997; Visscher 1999). Here we describe the use of *Cftr* intagenic markers which allowed fast and efficient identification of the differential locus during backcrossing. Moreover, this method provided a useful tool whereby unexpected events such as vector excision from the disrupted *Cftr* locus have been revealed posing questions for the stability of insertional mutants generated by this strategy. Furthermore, given our observations that different haplotypic backgrounds were found between the inbred strains raises questions on whether alleles at polymorphic loci can affect cftr at the transcript and/or protein level and whether it would be beneficial to study *Cftr* induced mutations on the respective haplotypic background of the individual strains.

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Chapter 3

Phenotypic characterisation of CF/1- $Cftr^{TgH(neoim)Hgu}$ and CF/3- $Cftr^{TgH(neoim)Hgu}$

Abstract

From the original C*ftr*$^{TgH(neoim)Hgu}$ mutant mouse model with a divergent background we have generated two inbred C*ftr*$^{TgH(neoim)Hgu}$ mutant strains named CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3-C*ftr*$^{TgH(neoim)Hgu}$ using brother x sister mating for more than 20 generations. Initial phenotypic characterisation on these two CF inbred strains indicated that they differed in the levels of correctly spliced *Cftr* mRNA production. Low amounts of wild type Cftr protein was detected for both strains in the intestine (CF/1- C*ftr*$^{TgH(neoim)Hgu}$ 20% of wt, CF/3- C*ftr*$^{TgH(neoim)Hgu}$ 10% of wt). Short circuit current measurements in the respiratory and intestinal epithelium revealed that, both strains have ameliorated the basic defect of cystic fibrosis with a presentation of a subnormal electrophysiology in both tissues. On the basis of these findings both CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ offer an excellent model whereby determination of the minimal levels of protein required for the amelioration of the basic defect of CF can be studied, along with the modulating factors which may affect this outcome.

3.1) Introduction

Cystic fibrosis (CF) is a fatal autosomal recessive disorder characterised by defective chloride transport in epithelial cells and excess mucus secretion (Davis *et al*., 1996). It is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*), resulting in defective cAMP-dependent chloride conductance (Riordan *et al*., 1989). Isolation of the mouse *Cftr* gene enabled the modelling of the disease in the mouse by gene targeting in embryonic stem cells. To date thirteen mouse models of CF have been reported in the literature, with varying degrees of characterisation. Mouse models generated using a replacement gene-targeting method to disrupt the *Cftr* gene (*Cftr*^{tm1Unc}, *Cftr*^{tm1Cam}, *Cftr*^{tm1Hsc}, *Cftr*^{tm3Bay}) (Snouwaert et al., 1992, Ratcliff *et al*., 1993, Rozmahel *et al*., 1996, Hasty *et al*., 1995) are absolute nulls with no wild type mRNA detectable. Models designed to mimic human clinical mutations depending upon the strategy used have either normal (double homologous recombination models, *Cftr*^{tm1Eur}, *Cftr*^{tm2Hgu}) (van Doorninck *et al*., 1995, Dickinson *et al*., 2002) or reduced levels (replacement gene-targeting models *Cftr*^{tm1Bay}, *Cftr*^{tm1Kth}, *Cftr*^{tm1G551D}) (O´Neal *et al*., 1993, Zeiher *et al*., 1995, Delaney *et al*., 1996) of mutant mRNA expression levels.

Dorin *et al.,* (1992), described a transgenic mouse model C*ftr*$^{TgH(neoim)Hgu}$ / C*ftr*$^{TgH(neoim)Hgu}$, generated following targeted insertional mutagenesis into exon 10 of the murine *Cftr* gene in embryonic stem cells. Unlike the *Cftr* mutants created by gene replacement low levels of wild type *Cftr* mRNA are produced as a result of exon skipping and aberrant splicing (Dorin *et al.,* 1994). Further characterisation of this mixed genetic background insertional mouse model indicated that it was viable, suffered from only mild intestinal obstruction with gut, lung and gonadal pathology characteristics of cystic fibrosis. Despite their residual levels of wild type mRNA C*ftr*$^{TgH(neoim)Hgu}$/C*ftr*$^{TgH(neoim)Hgu}$ mutant mice displayed the electrophysiological defect in the gastrointestinal and respiratory tract characteristic of CF and could be unequivocally distinguished from their non-cf littermates (C*ftr*$^{TgH(neoim)Hgu}$ /+ and +/+) on this basis (Smith *et*

al., 1995). This argued that even though there is production of wild type mRNA there can only be minimal functional Cftr protein sufficient to ameliorate the otherwise fatal intestinal obstruction but not enough to ameliorate the intestinal electrophysiological disease phenotype (Dorin *et al*., 1992).

From the original C*ftr*$^{TgH(neoim)Hgu}$ mutant mouse model (Dorin *et al*., 1992) with a divergent background we have generated two inbred C*ftr*$^{TgH(neoim)Hgu}$ inbred mutant lines named CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3-C*ftr*$^{TgH(neoim)Hgu}$ using brother x sister mating for more than 20 generations. Phenotypic evaluation of the inbred CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3-C*ftr*$^{TgH(neoim)Hgu}$ animals indicated that both lines have ameliorated the basic defect of CF with a presentation of a subnormal electrophysiology in both the intestinal and nasal epithelium. Unlike leaky splicing mutations in the human the trace amounts of correctly spliced mRNA and the subsequent low amounts of wild type Cftr protein substantiated for the amelioration of the disease phenotype in the two inbred C*ftr*$^{TgH(neoim)Hgu}$ strains. Based on this finding both CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3-C*ftr*$^{TgH(neoim)Hgu}$ offer an excellent model to determine the minimal levels of wild type Cftr required to ensure a normal chloride secretory capacity of the intestine preventing intestinal obstruction. Moreover, the mechanisms operating at the transcriptional and translational level to ameliorate lethal mutations can be addressed.

3.2) Materials and Methods

3.2.1) Experimental animals

All experiments were approved by the local Institutional Animal Care and Research Advisory Committee as well as by the local government. C*ftr*$^{TgH(neoim)Hgu}$ mice were bred under specified pathogen-free conditions in the isolator unit of the Central Laboratory Animal Facility of the Hannover Medical School. Mice were kept in a flexible film isolator. The temperature within the insulator was maintained at 20-24ºC with 40-50% relative humidity. Animals were fed an irradiated (50 kGy) standard chow (SSniff) and autoclaved water (134ºC for 50min) ad libitum.

3.2.2) Generation of inbred Cftr$^{TgH(neoim)Hgu}$ mutant mice.

For the gene targeting and germline transmission to generate the C*ftr*$^{TgH(neoim)Hgu}$ mice, the embryonal stem cell line E14 was used (Dorin *et al*., 1992). E14 originates from the inbred mouse strain 129 (subline 129/Ola-Hsd, abbreviated designation: 129P2). These cells were injected into blastocysts of the inbred strain C57BL/6. The resulting chimeric offspring was crossed to C57BL/6 and to the outbred strain MF1 for several times to increase reproduction.

The inbred population of C*ftr*$^{TgH(neoim)Hgu}$ mice was established from one pair with divergent genetic background of one homozygous male and one homozygous female at generation F4. Two separate litters were obtained and two animals of each litter became the starting population for the establishment of the two individual inbred C*ftr*$^{TgH(neoim)Hgu}$ lines CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3-C*ftr*$^{TgH(neoim)Hgu}$ which were generated by brother-sister mating for now more than 26 generations.

3.2.3) Isolation of RNA and RT-PCR.

Tissue samples from the intestine were immediately frozen in liquid nitrogen after extirpation and stored at -70ºC until use. Prior to RNA isolation, all solutions and plastic equipment were treated for at least 12 h with 0.04% aqueous diethyl pyrocarbonate solution. RNA was isolated from the homogenized tissue as described by Chomczynski *et al*., 1987. In brief, following isopropanol precipitation, the RNA pellet was precipitated with isopropanol and prepared for storage at -70ºC. The absence of contamination by DNA was verified by PCR. mRNA was converted to cDNA and subjected to amplification by PCR with Taq polymerase (InviTaq). For the detection of the correctly spliced mRNA the wild type mouse *Cftr* cDNA was amplified with a sence exon 9 primer 5´GCT ATT ACT GGA TCT ACT GGA CTA GG and an antisense exon 13 primer 5´GCT CGG ACG TAG ACT TTG TAG C. The PCR reaction was carried out at 58ºC annealing for 1 min, 72ºC extension for 1 min, and 92ºC denaturation for 30 sec. cDNA from MF1 wild type mouse were used as controls.

3.2.4) Sequencing.

Following RT-PCR cDNA products were sequenced by Qiagen GmbH.

3.2.5) Western Blot analysis.

Wild type MF1 mice and CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mutant mice were anaesthetized with a hypnorm/diazepam mixture. Their abdomens were opened and their small intestines dissected. Epithelial cells originating principally from the villus region were isolated at 0-4ºC from the jejunum by everting the intestinal segments on metal rods attached to a vibration apparatus (Vibromixer type E1 from Chemap A.G.) and exposing them to vibration (50 Hz, amplitude 1.5 mm) for 30 min in 0.14 M NaCl containing 5 mM EDTA pH 7.4. Detached jejunal enterocytes were collected by centrifugation at 800 g for 15 s and suspended in 10 ml of a medium containing 12 mM Tris-HCl pH 7.4, 0.3 M mannitol, 10 mM

KCl, 0.5 mM EDTA and a protease-inhibitor cocktail containing 0.3 mM Pefablock (Boehringer Mannheim Germany), 10 μg/ml aprotinine, 5 μg/ml leupeptine, 1 μg/ml pepstatin A, 1 μg/ml chymostatin, 50 μg/ml soybean trypsin inhibitor and 0.03 g/l phosphoramidon. Vesiculation of intestinal membranes was achieved by a freeze-thaw procedure described initially for rat enterocytes and crude microsomal membranes were isolated from half of the cell lysate by a two-step differential centrifugation procedure (10 min, 4000 g, followed by 60 min, 40 000 g). The other half was used to isolate BBMV by differential precipitation with 10 mM $MgCl_2$ and differential centrifugation (15 min, 3000 g followed by 30 min, 27 000 g) essentially as described by van Dommelen *et al*., (1986). The membrane pellets were solubilized by vortexing in 30 μl modified Laemmli sample buffer [0.06 M Tris-HCl; 2% (w/v) SDS, 10% (w/v) glycerol, 0.1 M dithiothreitol, 0.1% (w/v) bromophenol blue and the protease inhibitor cocktail, pH 6.8] and incubated for 30 min at room temperature. Following centrifugation (2 min, 8000 g) samples of the supernatant (10 μl, adjusted to 20 μg protein) were separated on 6% polyacrylamide slabgels using a Bio-Rad Miniprotean apparatus (Bio-Rad Laboratories). Proteins were subsequently electroblotted onto nitrocellulose paper (0.1 μm pore size; Schleicher and Schuell) in 0.025 M Tris, 0.192 M glycine, 20% (v/v) methanol. The blots were incubated overnight at 4ºC with 0.02 M Tris-HCl, 0.15 M NaCl, 0.1% (w/v) Tween 20 pH 7.5 (TTBS), followed by overnight incubation at 4ºC with a 1:1000 dilution of affinity-purified anti-CFTR antibody R3195 in TTBS. Blots were washed three times in TTBS, incubated with peroxidase-conjugated anti-rabbit IgG (Tago Inc.; 1:3000 in TTBS for 2 h), and washed four times with TTBS. Peroxidase activity was detected with bioluminescence reagent (ECL kit; Amersham) on X-ray film, and CFTR bands were quantitated with the Molecular Imaging System GS-363 (Bio-Rad).

3.2.6) CFTR antibody.

The rabbit polyclonal antibody R3195 was raised against a thyroglobulin-conjugated 13 amino acid COOH-terminal peptide sequence of rodent CFTR and was affinity purified on a peptide epoxide-activated Sepharose column, eluted with 4.9 M $MgCl_2$, dialysed and concentrated. CFTR labelling specificity has been demonstrated previously in western blot and immunocytochemical assays by the loss of immunostaining in tissue specimens from $CFTR^{-/-}$ mice (French *et al*., 1996).

3.2.7) Immunocytochemical analysis.

Wild type and mutant CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mice were sacrificed by cervical dislocation, the intestine was dissected and the jejunum, duodenum and ileum were rinsed with ice-cold saline and fixed in 3% (w/v) paraformaldehyde for 16 h, prior to standard paraffin embedding. Sections (5 μm) were deparaffinised, followed by microwave treatment in 0.01 M sodium citrate solution according to Devys *et al*., (1993) Endogenous peroxidase activity was blocked by a 30 min preincubation in 0.1 M PBS, 0.6% (v/v) H_2O_2 and 0.12% (w/v) sodium azide. Subsequently, sections were incubated with antibody R3195 (1:100) at room temperature for 1 h followed by a 45 min incubation with a peroxidase-conjugated secondary antibody. Enzymatic detection of antigen-antibody complexes was achieved by incubation in substrate solution containing H_2O_2 and 3,3´-diaminobenzidine tetrahydrochloride (DAB; Serva). Finally, the sections were counterstained with haematoxylin. Labelling specificity was verified by incubations without primary antibody. In both cases, background labelling appeared negligible.

3.2.8) Short current measurements.

Freshly excised mouse ileum and nasal epithelium of CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3-C*ftr*$^{TgH(neoim)Hgu}$ homozygous animals were used for short circuit measurements (SCC) and compared with wild type controls. Experiments were performed at 37ºC. The basic perfusion solution (modified Meyler`s solution) consisted of 105 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 10 mM HEPES, saturated with 95% O_2 and 5% CO_2, pH 7.4.

Mouse ileum was excised under hypnorm/diazepam anaesthesia and reverted on a plastic rod. The muscle layer was cut longitudinally using a blunt razor blade and was stripped of fat manually. The stripped tissue was mounted in a holder with the mucosal side up (exposed tissue area 0.2 cm^2). After insertion of the holder into the Ussing chamber the tissue was allowed to recover for 10-20 min and to reach a stable baseline. To the serosal side of the ileum glucose (10 mM) and indomethacin (10 mM) were added. After equilibrium, the following compounds were added to the mucosal (M) or serosal (S) side of the tissue: Forskolin (10 μM, S), genistein (100 μM, M+S) and carbachol (200 μM, S). All compounds were present throughout the experiment.

Mouse nasal epithelium was isolated as described by Grubb *et al*., (1994). In brief the mice were sacrificed by servical dislocation and the skin of the head was peeled back in order to reach the underlying paired nasal bones. These were removed and the two sheets of the nasal epithelia, separated by the septum, were isolated independently. The sheets of epithelia were mounted immediately between the Ussing chambers (exposed area 1.13 mm^2). The chambers were filled with gassed modified Meyler`s solution supplemented with glucose (10 mM) and indomethacin (10 mM) and the short circuit measurements were started. After equilibration amiloride (10 μM, M) was added followed by forskolin (10 μM, M after stabilization of the current).

3.2.9) CFTR immunostaining in mouse nasal epithelium.

For immunocytochemical staining of nasal epithelium the tissue was treated as described above. In case of immunofluorescent staining the sections were incubated with the primary antibody CFTR 3195 (1:100) for 1.5 h followed by the incubation with goat-anti –rabbit FITC (Nordic) labelled secondary antibody (1:100) for 45 min.

3.3) Results

3.3.1) Generation of the inbred CF/1- Cftr$^{TgH(neoim)Hgu}$ and CF/3- Cftr$^{TgH(neoim)Hgu}$ mutant mice.

From the original C*ftr*$^{TgH(neoim)Hgu}$ mutant mouse generated using insertional mutagenesis in the *Cftr* exon 10 (Dorin *et al*., 1992) we have established two inbred CF strains CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ by strict brother sister mating for now more than 26 generations.

3.3.2) Cftr mRNA analysis.

In order to determine whether CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3-C*ftr*$^{TgH(neoim)Hgu}$ animals produce wild-type Cftr mRNA, we used RT-PCR spanning exon 9 to exon 13, using MF1 wild type animals as positive controls. Wild type mRNA corresponded to a cDNA product of 555 bp in size. For the inbred line CF/1- C*ftr*$^{TgH(neoim)Hgu}$ faint but otherwise pure bands were detected corresponding to the expected appropriate size indicative of only trace amounts of correctly spliced mRNA in these mice. No signal was detected for the CF/3- C*ftr*$^{TgH(neoim)Hgu}$ inbred line arguing for either extremely reduced to absent production of correctly spliced Cftr mRNA in these animals.

Sequencing of the PCR products obtained for the CF/1- C*ftr*$^{TgH(neoim)Hgu}$ mutant mice revealed that the observed trace amounts correspond 100% to wild type *Cftr* cDNA sequence.

The previously described (Dorin *et al*., 1994) alternatively spliced mRNA encompassing the 206 bp fragment from the targeting vector pIV3.5H which is spliced between exon 9 and 10 was not detected in any of the samples examined in this study.

3.3.3) CFTR processing in CF/1- Cftr$^{TgH(neoim)Hgu}$ and CF/3- Cftr$^{TgH(neoim)Hgu}$ mutant mice.

Analysis of brush border membrane vesicle (BBMV) preparations consisting of apical membranes of mutant homozygous CF/1- *Cftr*$^{TgH(neoim)Hgu}$ and CF/3- *Cftr*$^{TgH(neoim)Hgu}$ mice demonstrated the presence of fully glocosylated isoform (band C) of CFTR in these mice

(Figure 1). The intensity of the C band of these mice was reduced when compared with the intensity of the C band of the wild type MF1 mice (n=2).

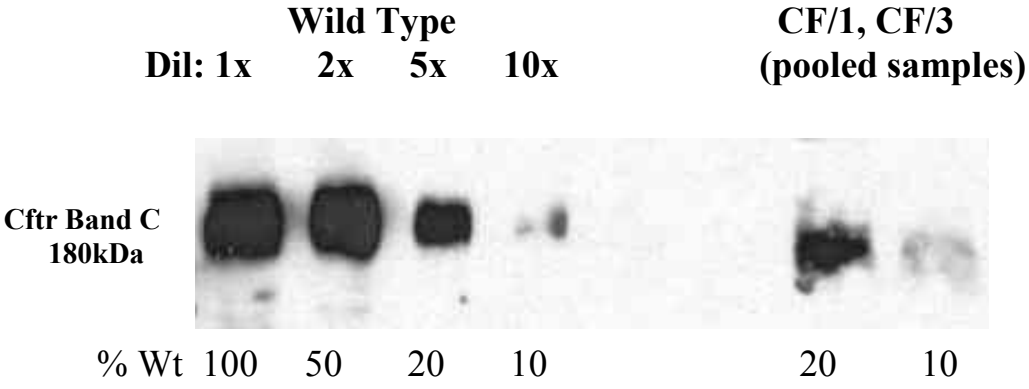

Figure 1. Quantification of wild type Cftr protein in apical membranes of CF/1- *Cftr*$^{TgH(neoim)Hgu}$ and CF/3- *Cftr*$^{TgH(neoim)Hgu}$ mutant mice. BBMV isolated from wild type MF1 mice were subjected to western blot analysis as described in Materials and Methods. Blots were labelled using the CFTR-specific R3195 antibody.

By running pooled samples of the CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mutant mice together with serial dilutions (5x, 10x, 20x, 50x, 100x) of membranes from the MF1 wild type mice the amount of mature CFTR (band C) in the two inbred mutant mice was estimated to be 20% of wild type for CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and 10% of wild type Cftr protein for CF/3- C*ftr*$^{TgH(neoim)Hgu}$.

3.3.4) Phenotype of the inbred CF1- C$ftr^{TgH(neoim)Hgu}$ and CF3- C$ftr^{TgH(neoim)Hgu}$ mutant mice.

Homozygous CF/1-C$ftr^{TgH(neoim)Hgu}$ and CF/3- C$ftr^{TgH(neoim)Hgu}$ mice do not show any increased mortality over wild type animals (pre-or post weaning) weight does not differ significantly from wild type (MF1) and both males and females are fertile.

Histological analysis of intestinal sections (jejunum, ileum, duodenum) revealed focal hypertrophy of goblet cells in the CF/1- C$ftr^{TgH(neoim)Hgu}$ and CF/3- C$ftr^{TgH(neoim)Hgu}$ homozygous mutant mice (Figure 2). This hypertrophy, is more severe than that observed in the $Cftr^{tm1Eur}$ and $Cftr^{tm2Hgu}$ mice.

3.3.5) CFTR immunocytochemistry.

Wild-type FVB mice showed strong apical Cftr-immunoreactive staining of crypts throughout the gastrointestinal tract whereby intensity decreased continuously from duodenum to terminal ileum. In inbred CF/1- C$ftr^{TgH(neoim)Hgu}$ mice staining with the R3195 Cftr antibody revealed weak and mainly diffuse signals with some apical labelling in the upper villus region of the ileum, whereas no specific immunoreactive signals could be detected in sections from duodenum and jejunum (Figure. 2). Sections of the intestine of CF/3- C$ftr^{TgH(neoim)Hgu}$ mice showed substantially weaker staining at the limit of detection.

Cftr immunostaining of nasal respiratory epithelium (Figure 3) yielded strong punctuated apical labelling of similar intensity in CF/1- C$ftr^{TgH(neoim)Hgu}$, CF/3- C$ftr^{TgH(neoim)Hgu}$ and FVB wild type, with the F508del homozygous mice exhibiting much fainter signals.

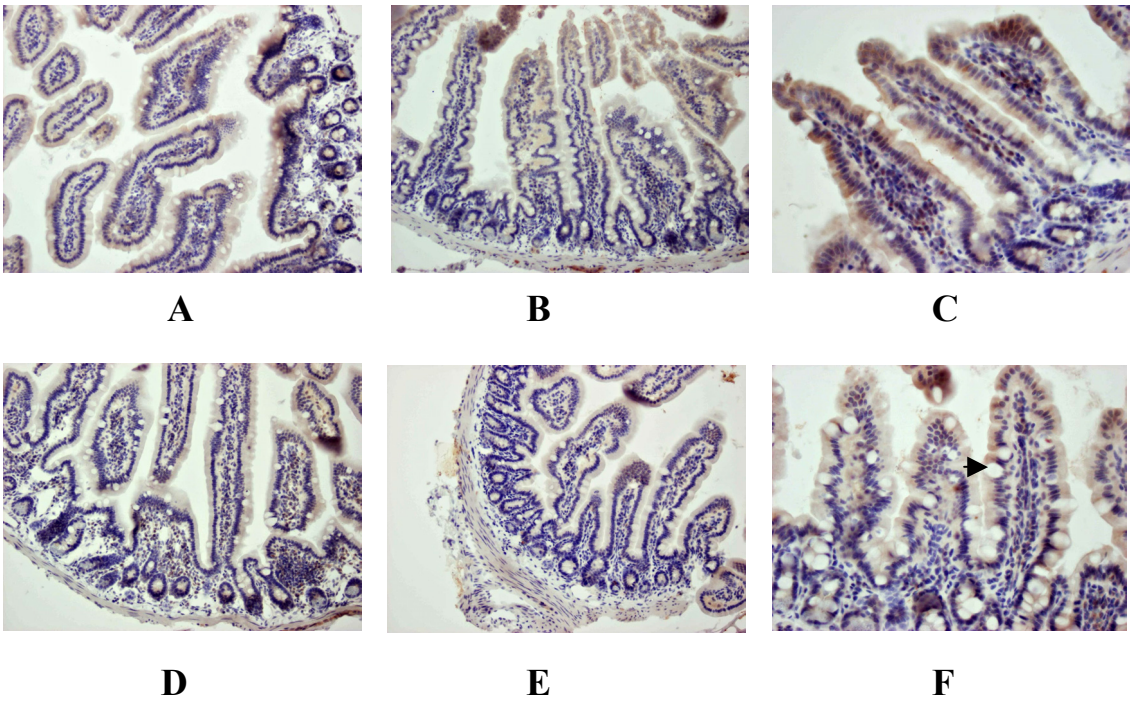

Figure 2. Immunohistological analysis of C*ftr*$^{TgH(neoim)Hgu}$ Cftr expression. Immunocytochemical staining of Cftr in the duodenum (A), jejunum (B) and ileum (C) of the CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and in the duodenum (D), jejunum (B) and ileum (C) of CF/3- C*ftr*$^{TgH(neoim)Hgu}$. Examples of goblet cells are indicated in black arrows.

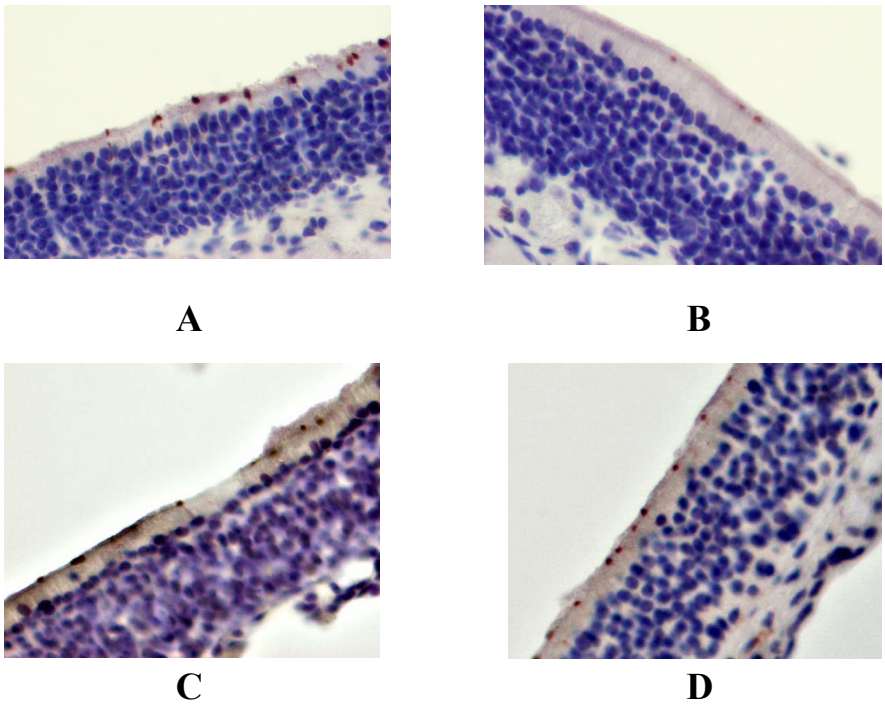

Figure 3. Cftr immunostaining in the nasal epithelium of a wild type FVB mouse (A), homozygous F508del (B), CF/1- C*ftr*$^{TgH(neoim)Hgu}$ (C) and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ (D).

3.3.6) Electrophysiological characteristics of CF/1-Cftr$^{TgH(neoim)Hgu}$ and CF/3-Cftr$^{TgH(neoim)Hgu}$.

The electrophysiological profile of CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mice both in the nose and the intestine was examined by current measurements of ion secretory responses in Ussing chambers under short-circuit conditions. The chloride secretory responses of homozygous CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mice were compared with those of wild type animals (FVB) and of *Cftr*^{tm1Eur} F508del homozygous mutant mice. Basal ion flow was similar in wild type and all examined CF strains (Figure 4). The chloride secretory response evoked by forskolin which activated the Cftr through an increase in cAMP amounted to about 50% of that observed in wild type animals and was significantly higher than that of F508del homozygous mice. The response of CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ to carbachol was intermediate between wild type and F508del homozygous mice. In contrast genistein which sustains the open state of CFTR channels generated larger short circuit current (SCC) currents in F508del than in CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3-C*ftr*$^{TgH(neoim)Hgu}$ mice.

In the nasal respiratory epithelium CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mice exhibited an intermediate phenotype between wild type and F508del mice with respect to short circuit current (SCC) (Figure 5) under basal conditions and exposure to amiloride which blocks sodium reabsorption. Interestingly, the forskolin cAMP- activated chloride secretory response was indistinguishable from wild type in all three examined CF models.

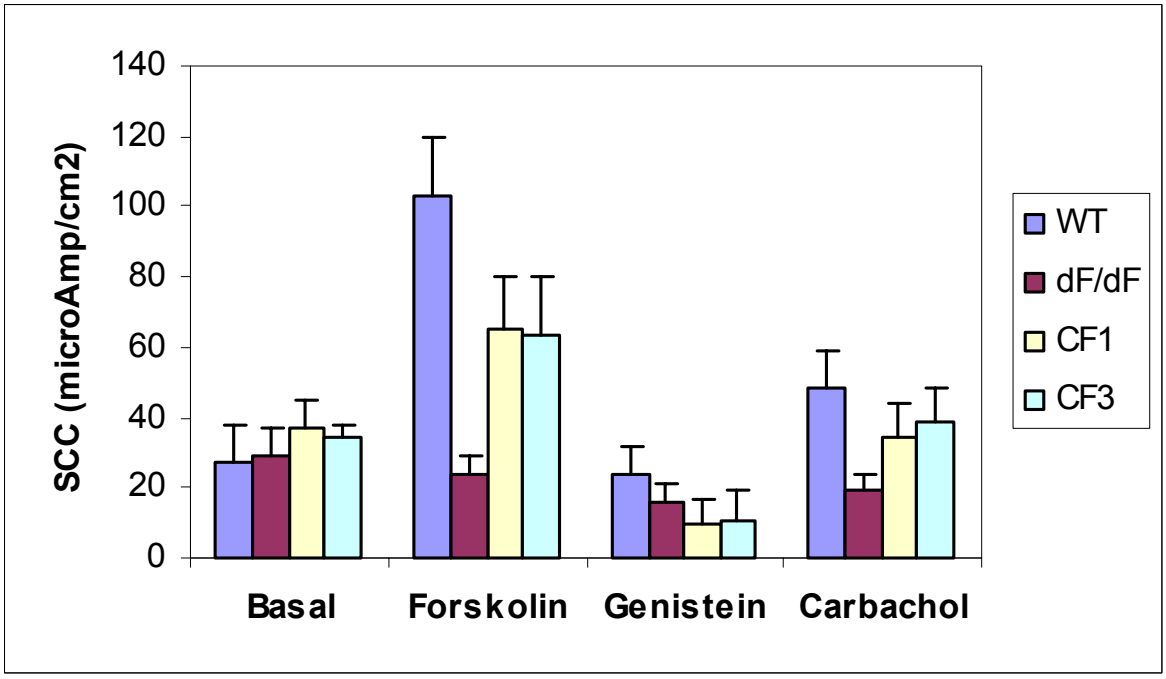

Figure 4. Bioelectric characteristics of the CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ in the ileum, compared with those of wild type and homozygous F508del mice.

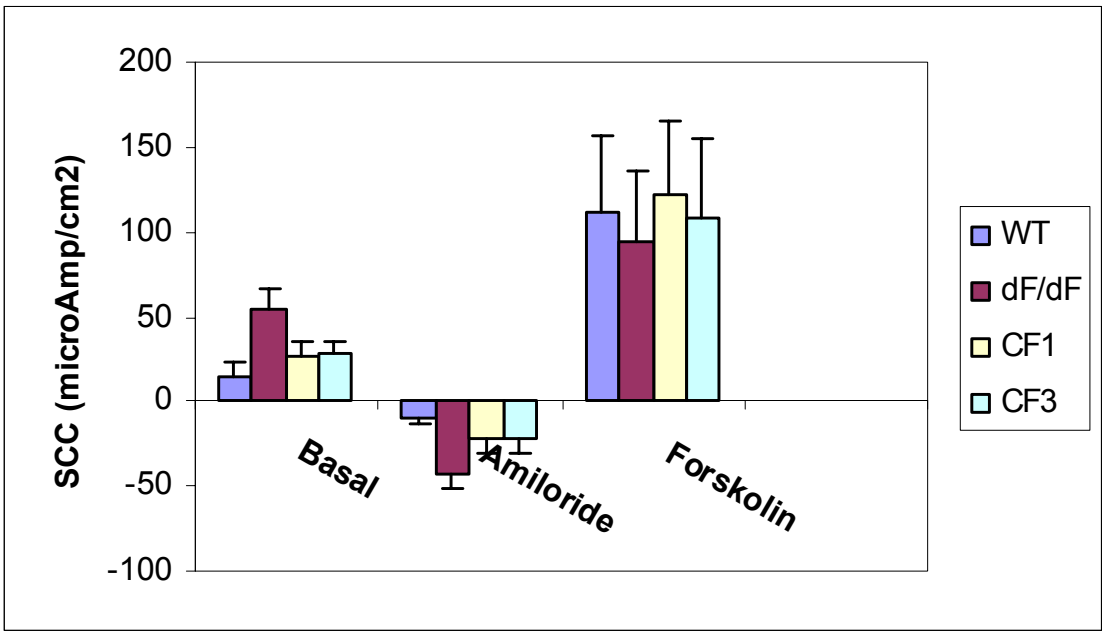

Figure 5. Bioelectric characteristics of CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ in the nasal epithelium compared with wild type and F508del homozygous mutant mice.

3.4) Discussion.

3.4.1) CF1- Cftr$^{TgH(neoim)Hgu}$ and CF3- Cftr$^{TgH(neoim)Hgu}$ express trace levels of wild type Cftr mRNA and low amount of protein

In agreement with a previous study on the outbred C*ftr*$^{TgH(neoim)Hgu}$ (Dorin *et al*., 1994) the expected mutant mRNA with an intact vector sequence was not detected in either of the two inbred strains. Moreover, the previously described (Dorin *et al*., 1994) novel mRNA encompassing a 206 bp fragment from the targeting plasmid vector pIV3.5H spliced between exon 9 and 10 was also not detected in either one of the two inbred strains.

RT-PCR from exon 9 to 13 revealed only trace levels of normal message in CF/1- C*ftr*$^{TgH(neoim)Hgu}$ homozygous CF animals but failed to detect any levels of correctly spliced Cftr mRNA in CF/3- C*ftr*$^{TgH(neoim)Hgu}$ (cf/cf) animals in the intestine.

Consistent with the mRNA analysis western blot analysis on BBMV revealed that CF/1- C*ftr*$^{TgH(neoim)Hgu}$ produce approximately 20% of wild type levels of Cftr protein, moreover the same analysis showed that CF/3- C*ftr*$^{TgH(neoim)Hgu}$ (cf/cf) animals produce wild type Cftr protein in their enterocytes which is approximately 10% of wild type and hence two fold reduced compared with CF/1- C*ftr*$^{TgH(neoim)Hgu}$. The latter indicated that even though mRNA levels were below the level of detection for the CF/3- C*ftr*$^{TgH(neoim)Hgu}$ animals, wild type protein is present indicating that splicing of the insertional mutation takes place in both inbred C*ftr*$^{TgH(neoim)Hgu}$ mouse strains.

3.4.2) Electrophysiological phenotype in the intestinal and nasal epithelium.

Despite the apparent absence of pulmonary pathology, electrophysiological analyses of the various CF mouse models have been able to differentiate between mutants and wild type littermates. Studies characterising the electrophysiological profiles of the intestines have found broadly the similar phenotypes in the different models. CF mouse models display a significant decrease in the baseline potential difference (PD) and short-circuit current (I_{sc}) in

the intestine. The mild nature of intestinal disease in *Cftr*^{tm1Eur} F508del and *Cftr*^{tm2Hgu} mice is less clear but is probably due to a significant fraction of protein that is able to escape the quality control mechanism in the ER and travel to the cell surface In the nose CF mouse models in common with individuals have a raised basal potential difference (PD) indicating a defect in sodium absorption.

Unlike mice with a complete disruption of *Cftr* expression, inbred homozygous CF1-C*ftr*$^{TgH(neoim)Hgu}$ and CF3- C*ftr*$^{TgH(neoim)Hgu}$ mutant mice do not suffer from intestinal blockage similar to *Cftr*^{tm2Hgu} and *Cftr*^{tm1Eur} mutant mice. CF1- C*ftr*$^{TgH(neoim)Hgu}$ and CF3- C*ftr*$^{TgH(neoim)Hgu}$ mutant mice do not show a defect in their forskolin response in the intestine with the baseline and carbachol response not altered compared to wild type. In the nose the forskolin response in both CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mice was not different from wild type. Overall the electrophysiological phenotype suggested that unlike their outbred progenitor the two inbred CF strains managed to ameliorate the basic defect by utilising mRNA levels greatly reduced from those described in the original report by Dorin *et al*., 1994.

3.4.3) Mechanisms of function.

3.4.3.1) Selection during inbreeding.

One can speculate that this phenotypic outcome is a direct result of selection during the inbreeding procedure. The selected mechanism responsible for this outstanding outcome should relate to those cellular processes which are involved in both the transcriptional and translational level influencing mRNA stability and protein processing in these mice. Moreover, the bioelectric phenotype between CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ regardless of the variation observed between the protein levels of the two inbred strains could be explained by the effect of modifier genes present in the genetic background of the two strains. Genome wide scan using microsatellite markers for every 20 cM showed that there is

an approximate 15% divergence between the two strains arguing for the existence of modifier loci responsible of rendering CF3-C*ftr*$^{TgH(neoim)Hgu}$ to have a better differential regulation compared to CF1-C*ftr*$^{TgH(neoim)Hgu}$.

3.4.3.2) At the level of transcription.

The introduction of the vector sequence introduces an unnaturally large exon in the murine *Cftr* gene. Vertebrate internal exons are usually between 50 and 400 nt long; exons outside this size range may require additional exonic and/or intronic sequences to be spliced into the mature mRNA. Bruce and Peterson (2001) using exon 4 of mouse polymeric immunoglobulin receptor gene (654nt) showed that for the efficient splicing of large exons a strong 5´splice site is necessary along with two exonic sequences and one evolutionary conserved intronic sequence in order for the exon to be spliced intact. Also, sequences that enhance cryptic splice site use must be absent from this large exon. In the case of the insertional vector pIV3.5H, used for the generation of the C*ftr*$^{TgH(neoim)Hgu}$, activation of such cryptic splice sites in the plasmid sequence gave rise to the novel 206bp exon (Dorin *et al*., 1994) providing an alternatively spliced mRNA. This alternatively spliced mutant mRNA contains an in-frame stop at a position less than half way into the message. Skipping of exons containing premature stop codons (PTCs) has been demonstrated in several human genetic diseases (Bach *et al*., 1993, Dietz *et al*., 1993, Gibson *et al*., 1993, Hull *et al*., 1994) whereby the outcome is an abnormally spliced mRNA induced by a PTC in the skipped exon. Urlaub *et al*., (1989) have suggested a nuclear-scanning model, in which specific intranuclear factors are able to scan the primary transcripts for premature termination codons (PTCs). In our case of the novel mutant mRNA, subjection to exon skipping will result in the alternative splicing of the new plasmid derived exon allowing for a wild type correctly spiced mRNA (Figure 6).

In the human the alternative splicing of many pre-mRNAs is affected by the intracellular concentrations of antagonistic splicing factors of the serine-arginine rich (SR) family and

hnRNPA1 (Zahler *et al*., 1993, Caceres *et al*., 1994, Muro *et al*., 1999). Pagani *et al*., (2000) showed that a protein complex containing SR proteins binds to the intronic splicing silencer (ISS) elements in the CFTR intron 9 and represses splicing of exon 9, an inhibitory effect on exon 9 splicing was also demonstrated for the splicing factor hnRNPA1. However, the comparison between the human and murine CFTR slice variants showed that in the mouse the alternative splicing of exon 9 is not evolutionary conserved (Delaney *et al*., 1993).

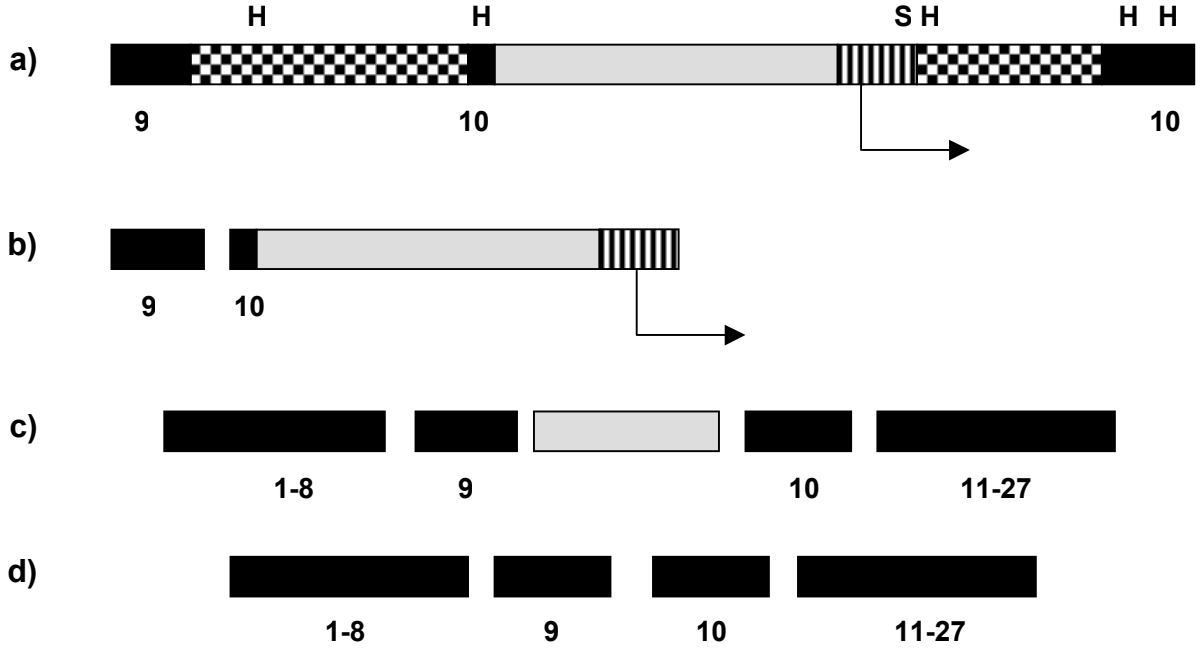

Figure 6. mRNA splicing. **a)** Structure of the insertional mutation in the *Cftr* gene. Black boxed represent exons, black and white squared boxes represent introns, gray boxes represent plasmid sequence, lines represent the neomysin gene. Abbreviation stand for: H: *Hind*III, S: *Sal*I.; **b)** predicted mRNA product from the mutant gene utilizing *neo* gene poly A addition signals; **c)** novel mutant mRNA deriving from the utilisation of cryptic splice sites in the plasmid sequence, resulting in a plasmid derived exon of 206 bp.; **d)** the sequence of the novel mRNA contains an in-frame stop at position less than half way into the message, exon skiping of the plasmid derived exon will result in wild type Cftr mRNA sequence.

3.4.3.3) Efficient protein processing mechanism.

The phenotypic observations provide evidence for the existence of a more powerful protein processing mechanism in both CF1- C*ftr*$^{TgH(neoim)Hgu}$ and CF3- C*ftr*$^{TgH(neoim)Hgu}$. Wild type CFTR matures inefficiently, as less than 30% of the protein produced in heterologous expression systems is converted into its fully glycosylated form (Ward *et al*., 1994) and it has been shown to undergo degradation through the ubiquitin-proteosome pathway (Ward *et al*., 1995). In vivo studies have suggested that molecular chaperones facilitate CFTR folding and maturation, namely heat-shock cognate (Hsc) 70/Hsp 70 (Yang *et al*., 1993), human DnaJ homologue (Hdj)-2 (Meacham *et al*., 1999) and Hsp90 (Loo *et al*., 1999) in the cytoplasm, and calnexin (Pind *et al*., 1994). Co-expression of Hsp70 and its co-chaperon Hdj-1/Hsp40 results in the stabilisation of the endoplasmic reticulum (ER) immature form of wild type CFTR, however over expression produces no significant effect on the conversion of the immature form of wild type CFTR into its fully glycosylated form. Based on these observations we can not exclude the possibility that such chaperones may play a key role in the stabilisation of the low levels wild type Cftr protein in the two inbred mouse strains CF1-C*ftr*$^{TgH(neoim)Hgu}$ and CF3- C*ftr*$^{TgH(neoim)Hgu}$ at the early stage of Cftr biogenesis, distinct from the events that cause its escape from the endoplasmic reticulum.

Furthermore, elimination of the mutant mRNAs will result in the accumulation only of the reduced amounts of correctly spliced mRNA preventing endoplasmic reticulum overload response (EOR), which is initiated by the accumulation of wild-type or misfolded proteins in the ER (Pahl 1999). This reduction in the ER stress may result in a more efficient processing of the wild type Cftr message.

3.4.3.4) Efficient Cftr protein trafficking and improved functional capacity at the apical membrane.

The forskolin-genistein induced CFTR-mediated chloride current for CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ in the small intestine was reduced to about 50% of wild type level, implying that about 10% of mature wild type Cftr protein in the apical membrane is required to ensure subnormal transepithelial intestinal currents providing evidence that both mutant mice have higher efficacy compared with the wild type to generate functional protein in their apical membrane. This could be the result of a) efficient Cftr trafficking from the post-ER compartment to the apical membrane resulting in a higher proportion of mature protein localisation in the apical membrane compared to the subapical compartment; b) higher functional capacity of the Cftr channels in the apical membrane.

a) Cftr trafficking. Various components have been shown to influence maturation and trafficking of CFTR from the ER/Golgi compartment to the basolateral and apical compartments i.e. COPI-coated vesicles (Orci *et al*., 1997); PDZ-containing proteins such as Csp (Zhang *et al*., 2002), EBP50 (Yun *et al*., 1997, Lamprecht *et al*., 1998), E3KARP (Yun *et al*., 1997), CAP70 (Wang *et al*., 1999), CAL (Cheng *et al*., 1999), SNARE proteins (Low et al., 1998), BAP31 (Lambert *et al*., 2001) and numerous Rab proteins. Early immunocolocalisation and surface biotinylation studies in T84 colonocytes and Chinese hamster ovary (CHO) cells stably transfected with CFTR showed that approximately 50% of the total pool of mature CFTR in the cell is localised at the plasma membrane and 50% localised intracellularly, suggesting the existence of a large subapical CFTR pool (Lukacs *et al*., 1992). The functional phenotype of CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ provide evidence that endocytosis and recycling of Cftr between the plasma membrane and intacellular compartments in the two strains is regulated so that a higher proportion of mature Cftr is targeted at the apical membrane. Endocytic recycling of membrane proteins is an important process that contributes to the fine tuning of membrane protein activities due to

environmental responses. The early endosomes are thought to be an important sorting station whereby proteins are targeted to the late endosomes or recycled back to the plasma membrane. Lukacs *et al*., 1997 demonstrated that budding prevention of clathrin-coated vesicles resulted in a depletion of the CFTR content of early endosomes. Webster *et al*., 1994 showed that in the ductal epithelium of submandibular glands, CFTR is co-localised with Rab4 which is a regulator of membrane traffic from early endosomes back to the apical membrane. A higher proportion of mature Cftr in the apical membrane compartment in CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mice could be due to a vesicle distribution of Cftr throughout the cell whereby targeting to the late endosomes is avoided and premature lysosomal degradation is reduced.

b) Activation of Cftr chloride channels. In agreement with the differential effect of genistein on F508-CFTR channels (Hwang *et al*., 1997) the forskolin-genistein induced CFTR-mediated chloride current observed for the *Cftr*^{tm1Eur} F508del homozygous mutant mouse model was increased compared to CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$. Moreover, the forskolin-genistein induced chloride conductance suggests that even though there is a lower estimated channel density in the CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ compared to wild type in the apical membrane the proportion of active channels i.e. at an open state, is higher compared to wild type. It is believed that protein kinase A (PKA) activates CFTR by phosphorylating multiple serine residues in the R domain (Wang *et al*., 2000). The partially phosphorylated CFTR channel has a lower open probability (Po) with characteristic prolonged closing times (Hwang *et al*., 1994). Wang *et al*., 1998 demonstrated that when the CFTR channel is strongly phosphorylated, the Po is increased with a concomitant shortening of the closed time. Phosphorylation of the regulatory domain is accomplished primarily by elevation of cAMP and activation of PKA therefore if increased Cftr phosphorylation is to be considered as a factor influencing the higher efficacy of the Cftr channels in CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ it would require an increase in

the levels of cAMP with the subsequent activation of the co-expressed β_2 adrenergic receptor which increase cAMP levels.

Following phosphorylation by PKA, the opening and closing of phosphorylated CFTR channels is coupled to ATP hydrolysis at NBD1 and NBD2 (Gadsby and Nairin 1999). Electrophysiological evidence supports the hypothesis that hydrolysis of ATP is essential for normal opening and closing or gating of the channel (Li *et al*., 1996). Several models have been proposed to account for the regulation of channel gating by ATP hydrolysis (Carson *et al*., 1995, Gadsby and Nairin 1994, Gunderson and Kopito 1995). An important point from all the models proposed is that there is an irreversible cycle of gating due to the high free energy of ATP binding and hydrolysis and that ATP concentration is an important determinant of the closed time between bursts of activity. Therefore, in the case of CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ the adaptive mechanism influencing increase of the Cftr channel activity i.e. the open probability (Po), is also linked to high available levels of ATP in these two mice. During inbreeding selection of breeding partners was based on the selection of those animals that were capable of producing sufficient litters and showed no severe disease symptoms. It is therefore plausible to assume that during the repeated cycles of inbreeding a mechanism may have evolved with a direct impact on the increase of ATP concentrations in the two strains. Such a mechanism should involve those signal transduction pathways which have a direct impact on the increase of energy metabolism in the two strains with a subsequent increase in the ATP stores.

3.4.5)Comparison with the human.

Unlike with what is seen in the CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mouse models in the human the amount of correctly spliced mRNA produced by apparently leaky alleles is not enough in order to ameliorate the disease severity in such a successful manner. Mutations affecting the splicing of the cystic fibrosis transmembrane conductance regulator (*CFTR*)

gene that lead to the simultaneous presence of correctly and aberrantly spliced transcripts cause CF disease due to insufficient levels of the normal mRNA, and hence, functional protein. Studies have reported a wide range of normal CFTR transcripts, from less than 4% to less than 20% (Chu *et al*., 1993, Rave-Harel *et al*., 1997), associated with "leaky" splicing mutations in the human. For the 3849+10kb C>T mutation, a CF allele associated with mild lung disease, pancreatic sufficiency and male infertility Highsmith *et al* (1994) reported 8% of normal CFTR mRNA. The mutation 2789+5G>A, resulting in a similar pulmonary and pancreatic phenotype but with male infertility has been reported (Highsmith *et al*., 1997) to be associated with lower levels of correctly spliced CFTR mRNA (4%). Whereas Ramalho *et al*., 2002 postulated that 5% of full-length CFTR mRNA in patients with 3272-26A>G/F508del is enough to ameliorate the severity of CF disease, because in these patients most clinical symptoms, including lung function and pancreatic involvement, are attenuated by comparison to patients with two severe mutations.

Several reasons could attribute to this observed discrepancies 1) Both 3849+10kb C>T and 2789+5G>A alleles produce alternatively spliced transcripts which bear premature termination signals. Nonsense mediated decay (NMD) a process by which cells recognise and degrade nonsense mRNAs to prevent possibly toxic effects or truncated peptides, enhances the degradation of messengers bearing premature stop codons in a process which is also described for CFTR transcripts (Will *et al*., 1995). Therefore one has to discriminate between the amount of different mRNA species produced and how these are being further processed; 2) Moreover simultaneous processing of the mutant protein along with the wild type CFTR will result in the increase of the ER stress because of the accumulation of misfolded proteins.

3.5) Conclusion

This study demonstrates that low amounts of protein can substantiate for subnormal phenotypic outcome in two inbred of CF strains. This amelioration of the basic defect defers substantially from the phenotype reported for the outbred C*ftr*$^{TgH(neoim)Hgu}$ whereby aberrant splicing of the construct and subsequent production of wild type *Cftr* mRNA did not substantiate for a correction in the electrophysiological phenotype indicating the existence of a modulating factor which has evolved during the inbreeding procedure in these two strains. Overall unlike any other mouse model described so far CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3-C*ftr*$^{TgH(neoim)Hgu}$ mutant mice provide a very good model whereby modulating factors involved in the amelioration CF disease at the level of transcription and protein processing and trafficking can be studied.

Chapter 3- References

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Chapter 4

Microsatellite genotyping of the two CF inbred strains (CF1-*Cftr*$^{TgH(neoim)Hgu}$ and CF3- *Cftr*$^{TgH(neoim)Hgu}$) and the three inbred strains (BALB/c, C57BL/6, DBA/2J).

4.1) Introduction

4.1.1) The laboratory mouse

One of the advantages working with mice is the availability of a large variety of *inbred* strains. The commonly known inbred strains originated almost exclusively from the fancy mice purchased by geneticists from pet mouse breeders like Abbie Lathrop and others at the beginning of the 20th century (Silver 1995)**.** Fancy mice had their origins from native animals captured in Japan, China, and Europe. Laboratory strains have a mosaic genome deriving from more than one species (Wade *et al*., 2002) and today's classical laboratory strains are regarded as interspecific recombinant strains deriving from four parental components: *Mus. m. domesticus* (Western Europe), *Mus. m. musculus* (Eastern Europe), *Mus. m. castaneus* (Southeast Asia) and *Mus. m. molossinus* (Japan). *Mus. m. molossinus* is thought to be a hybrid between *castaneus* and *musculus.* An inbred strain is considered to be genetically uniform (isogenic) and with the use of the same standard inbred strain it is possible to eliminate genetic variability as a complicating factor.

Strain	Color	Origin and Characteristics
BALB/c	Albino *A, b, c*	Stock acquired by H.Bagg in 1913, to MacDowell, to Snell in 1932 (who added the /c). Widely distributed and among the 2-3 most widely used inbred strains. Used in immunological studies, well known for the production of plasmacytomas on injection with mineral oil. Good breeding performance and long reproductive life-span. Data on genetic markers suggest that substrains have divergent largely through mutation or residual heterozygosity rather than genetic contamination.
C57BL/6	Black *a*	Substrain of C57BL (Little 1921) separated prior to 1937. Standard strain for genetic studies. It carries a Y chromosome of Asian *Mus musculus* origin (AKR and SWR) (Tucker et al 1992). And a LINE-1 element derived from *Mus spretus* the frequency of which suggests that up to 6.5% of the genome may be of *M. spretus* origin (Rikke et al 1995). Good reproductive performance.
DBA/2	Dilute Brown *a, b, d*	Substrain of the oldest of all inbred strains of mice (dba, Little 1909). Differences between the substrains are probably too large to be accounted for by mutation, and are probably due to substantial residual heterozygosity following dichotomy of the strains. Poor breeding performance with small litter size.

Table 1. Origin and characteristics of the three laboratory inbred strains used in this study.

4.1.2) Generation of inbred mice

Most breeding experiments usually start with an *outcross*, which is defined as the mating between two animals or strains considered unrelated to each other. All members of an inbred strain are homozygous across their entire genome and genetically identical to each other. The outcross between two unrelated inbred strains will result in offspring called the *first filial* generation (F_1) and will be identical to each other with a heterozygous genome. The offspring that will result from the mating of two F_1 siblings will produce the second filial generation. Unlike the F_1 where all animals are identical to each other (when generated from a pair of inbred strains), the animals in the F_2 which result from an *intercross* three different genotypes will be possible at every locus. At each subsequent filial generation, genetic homogeneity among siblings will be slowly recovered in a process which id referred to as *inbreeding*. This process leads to the production of inbred animals which will be genetically homogeneous and homozygous at all loci. The *International Committee on Standardized Nomenclature for Mice* has ruled that a strain of mice can be considered inbred at generation F_{20} (Silver 1995).

Outbred or *random bred* mice such as the MF1 or CD1 are sometimes used because they exhibit hybrid vigor with long life spans, high disease resistance, early fertility, large and frequent litters, low neonatal mortality, and rapid growth. The F_1 offspring of an outcross between an outbred and an inbred mouse will not be identical with one another. However, at each subsequent filial generation, genetic homogeneity among siblings will be recovered during the course of inbreeding.

4.1.3) Genetic background of the cystic fibrosis mouse models

Mouse models are used extensively in biomedical research. A major boost to the cystic fibrosis disease research was given by the generation of various mouse models using gene targeting in embryonal stem cells. Inbred mice which have been commonly used for backcrosses are the C57BL/6 (*Cftr*^{tm2Hgu}, *Cftr*^{tm1Kth}, *Cftr*^{tm2Cam}, *Cftr*^{tm3Bay}, *Cftr*^{tm1Bay}, *Cftr*^{tm1Unc},

Cftr^{tm1Cam}) (Dickinson *et al*., 2002, Zeiher *et al*., 1995, Colledge *et al*., 1995, Hasty *et al*., 1995, O´Neal *et al.*, 1993, Snouwaert *et al*., 1992, Ratcliff *et al*., 1993), the BALB/c (*Cftr*^{tm1Unc}) (Snouwaert *et al*., 1992) the FVB/N (*Cftr*^{tm1Eur}) (van Doorninck *et al*., 1995). Outbred mice have also been used in some models instead of inbred strains, such as the CD1 (*Cftr*^{tm1Hsc}, *Cftr*^{tm1G551D}) (Rozmahel *et al*., 1996, Delaney *et al*., 1996) and the MF1 (*Cftr*$^{TgH(neo\ im)1Hgu}$, *Cftr*^{tm1Cam}) (Dorin *et al*., 1992, Ratcliff *et al*., 1993).

4.1.4) Generation of congenic CF mouse models

The same *Cftr* mutations have been established on different inbred strains generating *congenic* strains, introducing the possibility of comparing the effects of genetic differences between the inbred strains and has facilitated the search for modifier genes.

Mice that have been bred to be *essentially* isogenic with an inbred strain except for a selected *differential chromosomal* segment are called *congenic* strains. Congenic production utilises in most cases the method known as the backcross or NX system. An outcross between the *recipient inbred partner* and the animal that carries the *donor allele,* which can be either an inbred or an outbred animal homozygous at the locus of interest, will result F_1 animals 100% heterozygous at all loci, in the case of an inbred partner. The second generation cross and all those that follow are backcrosses to the recipient inbred strain. At each generation (N_n) the offspring which carry the donor allele at the *differential locus* will be selected for the next backcross. By the tenth generation (N_{10}), the new strain is considered to be congenic (99%) (Silver 1995).

Initial characterisation of the different CF mouse models did not put emphasis on the background strain and its key role in phenotypic modification. The original study (Rozmahel *et al*., 1996) on the mixed *outbred* F_2 CD1/129-*Cftr*^{tm1Hsc} animals differentiated the animals in three different classes (Class I, II, III) depending upon when the mice died. Genetic experiments can not be performed straight forward with an outbred strain such as the CD1

since they are genetically non identical. To test the hypothesis of an unlinked modifier locus the original founder mouse was backcrossed to three different inbred strains (C57BL/6J, BALB/c, DBA/2J) generating *congenic* animals. Mortality varied significantly between the three different inbred backgrounds and the candidate modifier locus was mapped (using linkage analysis) on the proximal region of mouse chromosome 7 using dinucleotide repeats (*microsatellites*).

4.1.5) Microsatellite markers

Microsatellites are small blocks of tandemly repeated DNA, in which the repeated element is usually a di-, tri-, or tetranucleotide sequence. The class found most often in the mouse genome contains a $(CA)_n$ $(GT)_n$ dimmer. The number of repeat elements in these blocks is highly polymorphic, and shows simple Mendelian inheritance. Allelic variation is based on differences in the number of repeats present in a tandem array and the only way in which alleles can be distinguished is by measuring the total length of the microsatellite. Genotyping of microsatellites is accomplished through PCR amplification with primers on the flanking sequence on either side of the repeat, followed by denaturing gel electrophoresis

Microsatellites can be selected either manually by searching through sequence databases for a $(CA)_n$ repeat and design flanking primers or select primers for known polymorphic microsatellites via the Mouse Genome Database (www.informatics.jax.org).

4.1.6) Genetic relationships between inbred strains

Establishment of the genetic profile of inbred strains can be used for the determination of genetic relationships between different inbred strains. Inbred strains of mice are valuable model organisms also for studies in evolutionary biology, particularly at the molecular level (Atchley and Fitch 1991), as the genealogy of a number of genetically highly different strains is known with considerable certainty. Knowledge of the genealogical relationships and of the levels of genetic similarity helped researchers to evaluate the relative efficacy of various

phylogenetic algorithms, to determine the relative information content of various types of data and examine other biological phenomena by using taxa of known degrees of relatedness. Moreover the extensive genetic data available at different levels of organisation that can be used for phylogenetic analyses provided scientists with a useful tool with which evolutionary studies can be efficiently carried out. These data include DNA sequences, simple-sequence-length polymorphisms, restriction fragment length polymorphisms (RFLPs) mitochondrial DNA (mtDNA), X- and Y-chromosome sequences (Dietrich *et al*., 1992, Tucker *et al*., 1992, Atchley *et al*., 1993). Determination of the genealogical relationships among the available strains of mice has been difficult. This is because during the early history of inbred mice, mice from a small number of stocks held in a few laboratories (Cold Spring Harbor Laboratories, Bussey Institute, Rockefeller Institute) had probably contributed considerably to the genomes of the present day inbred strains of mice (Atchley *et al*., 1993). Determination of phylogenetic affinities among inbred strains is becoming complicated by the fact that many inbred strains include genetic components from different species, i.e., *Mus musculus*, *M. m. domesticus* and *Mus. m. castaneus* (Bonhomme *et al*., 1986, Tucker *et al*., 1992).

It can be important to have knowledge of genetic distances between strains, for choosing the best possible stains for an experiment. Phenotypic differences between inbred strains need to be taken into account when designing experiments. Strains may vary in characteristics that may not be relevant to the phenotype being studied, but which may indirectly influence experimental results. It is critical to have comprehensive data on the characteristics of the background strain before assessing the phenotypic consequences of introducing mutations. The relationships of the inbred strains of mice have been investigated using parsimony analysis by Atchley and Fitch (1991), employing classical genotypes at 144 loci over the whole genome. At each locus, possession by two strains of the same-sized allele indicates probable similarity by descent of that sequence. Microsatellite data lend itself conceptually to parsimony analysis because alleles differ by discrete steps (Weber *et al*., 1993, Amos *et al*.,

1996, Primmer *et al*., 1998). Compared to methods based on a distance matrix, parsimony allows more information to be considered in the analysis (Schalkwyk *et al*., 1999). The Wagner parsimony method does not assume that the ancestral state is known or that mutation in one direction is more probable than the other

4.2) Materials and Methods

4.2.1) Animals

All animals used in this study were bred under specified pathogen-free conditions in the isolator unit of the Hannover Medical School Laboratory Animal Facilities. Mice were kept in a flexible film isolator with a temperature maintained at 20-24ºC with 40-50% relative humidity. Animals were fed an irradiated (50 kGy) standard chow (Altromin 1314) and autoclaved water (134ºC for 50min) ad libitum.

All experiments were approved by the local Institutional Animal Care and Research Advisory Committee and by the local government.

4.2.2) Generation of inbred $Cftr^{TgH(neo\ im)1Hgu}$ mutant mice.

For the establishment of the inbred population of $Cftr^{TgH(neoim)1Hgu}$ mice a mixed background pair (generation F_4) of one homozygous male and one homozygous female was obtained from the MRC Human Genetics Unit, Edinburgh. Two separate litters were obtained and two animals of each litter became the founders of the two individual inbred $Cftr^{TgH(neoim)1Hgu}$ strains CF/1- $Cftr^{TgH(neoim)1Hgu}$ and CF/3- $Cftr^{TgH(neoim)1Hgu}$ which were generated by strict brother-sister mating for more than 26 generations.

4.2.3) Microsatellite selection

The primers flanking the microsatellite regions were obtained from the MGI database (www.informatics.jax.org). Seventy eight microsatellite markers with at least two different variants between the three inbred mouse strains, C57BL/6, DBA/2 and BALB/c were chosen via the MGI database at about every 20cM. Polymorphic loci between the two inbred CF strains were genotyped with markers flanking the informative loci in order to map the regions of genetic heterogeneity. In total 105 misrosatellites were chosen for the 19 autosomal chromosomes (Figure 1).

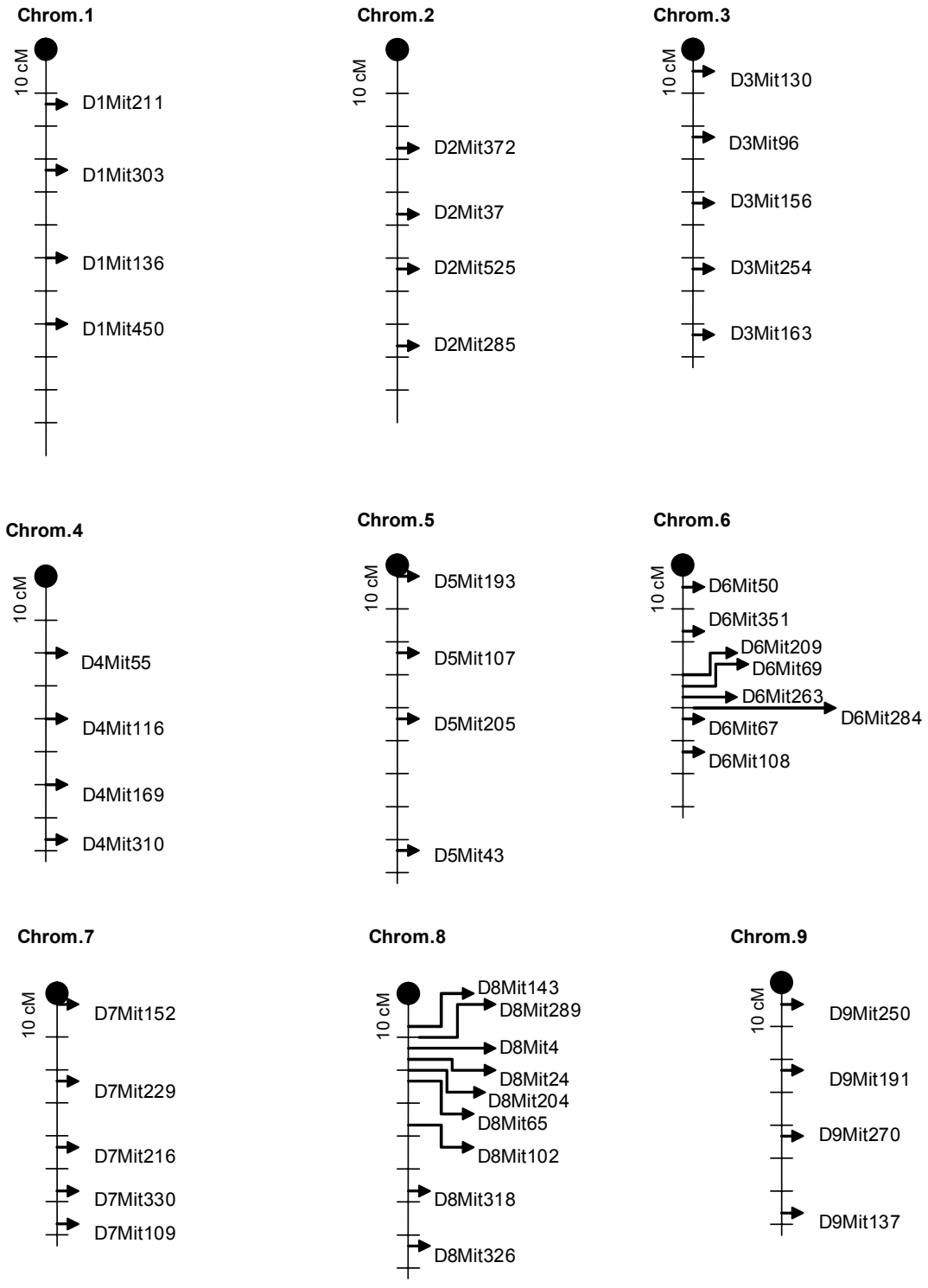


Figure 6. Location of the SSLP typed in this study. Names of markers are abbreviated following the rules and guidelines of the International Committee on Standardised Genetic Nonmenclature for mice (www.informatics.org/nomen) whereby DNA segments are symbolised according to the laboratory identifying or mapping the segment as "DNA segment, chromosome N, Lab name" and a serial number, where N is the chromosomal assignment (1-19) and is symbolised as DNLabcode.

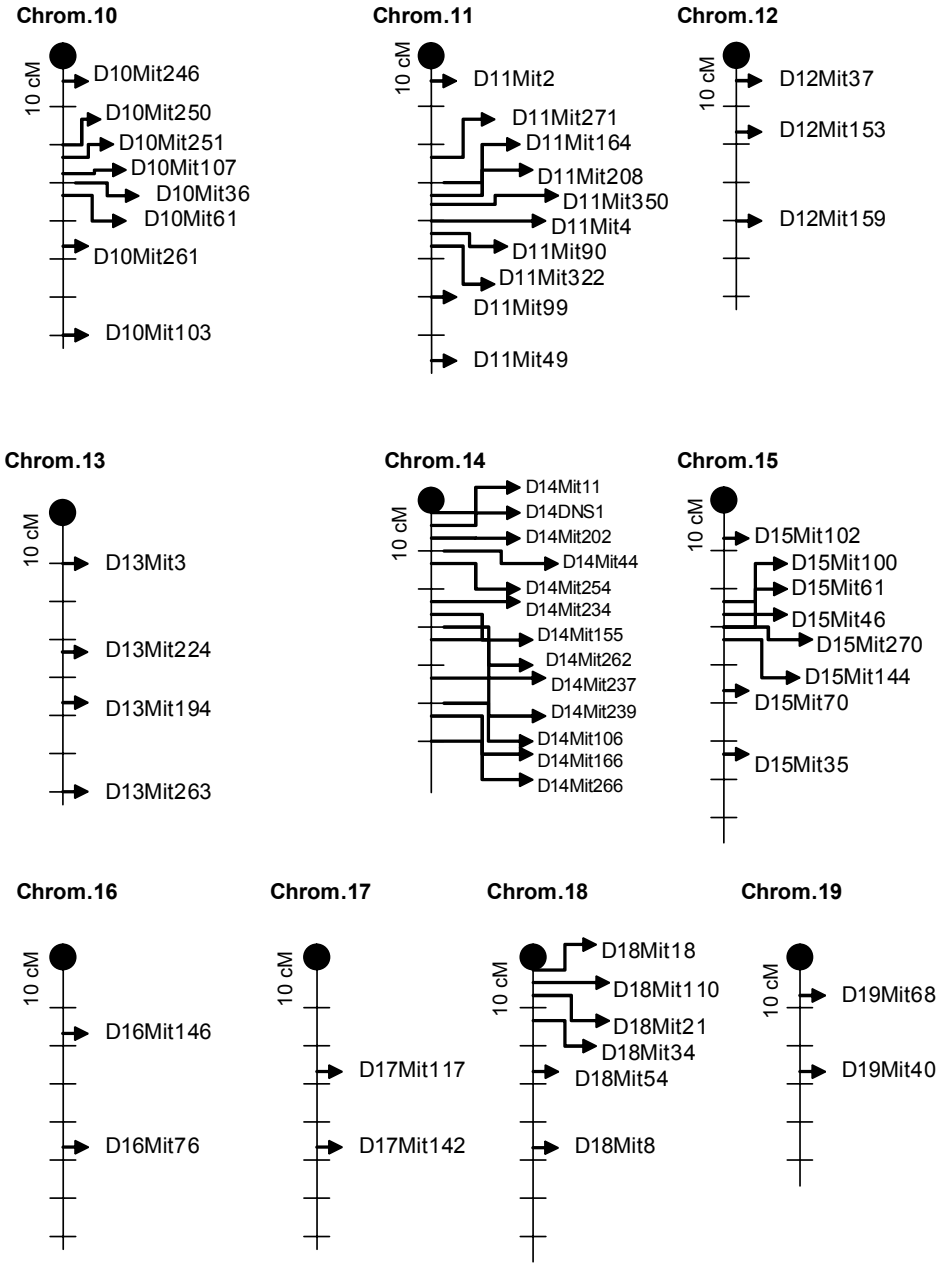


Figure 6 (cont) For example: D13Mit3, is the 3rd locus mapped on chromosome 13 by M.I.T. For one marker the laboratory designation is different: D14DNS1. Map scale is shown to the side of each chromosome. Chromosomes 6, 8, 10, 11, 14, 15 and 18 were densely genotyped in order to map the regions of genetic heterogeneity between CF/1 and CF/3.

4.2.4) High molecular weight DNA isolation

High molecular weight DNA was isolated from spleen tissue (from ten CF/1, ten CF/3, two BALB/c, two C57BL/6 and two DBA/2) either fresh or thawed on ice after storage at -20C based on the protocol described by Gross and Bellard *et al*.(1973). The tissues were incubated with 50ml lysis buffer (50mM Tris-HCl, 109.5g/l Saccharose, 1% w/v Triton X-100) for 30min on ice followed by centrifugation at 5000 x g for 15 minutes. The pellet was treated with 40µl of 50mg/ml proteinase K, 200µl SDS 10% in STE and was allowed to incubate for 12 hours at 56ºC in a shaking water bath. Digested proteins and high molecular weight DNA were separated two times with phenol/chloroform extraction: 3ml phenol and 3ml chloroform/isoamylalchohol were added to the digest followed by a gentle mix for 10 minutes, centrifugation at 5000 x g for 10 minutes, and removal of the organic phase. This extraction step was followed by a two times extraction with 6ml chloroform in order to remove phenol remaining in the aqueous phase. DNA was precipitated by adding 1/10 of the volume of the aqueous phase of 3M sodium carbonate (pH 5.5) and 30ml of 70% ethanol (-20C). High molecular weight DNA was transferred into an Eppendorf cup containing 1ml of 70% ethanol and was further centrifuged at 13000 x g for 3 minutes. The resulting pellet was washed with 70% ethanol two times and was allowed to dry at 37C for no more than 30 minutes, and dissolved in 1ml TE buffer at 4ºC.

4.2.5) Genotyping of microsatellites

4.2.5.1) PCR

All microsatellite markers were genotyped using PCR in 96 well plates (multiwell plates) purchased from Greiner, Frickenhausen, precoated with 50ng of DNA template per well in a Hybaid Thermocycler (Hybaid, Teddington) with a heated lid. One of the two primers per microsatellite was 5´-terminal biotinylated and every PCR reaction was performed in a total volume of 30µl, without oil overlay using InViTaq polymerase (InViTek, Berlin).Evaporation

during the reaction was prevented with the use of PCR foil purchased by Eppendorf covering the multiwell plate

4.2.5.2) Analysis of polymorphisms using direct blotting electrophoresis

After PCR an 8µl aliquot was transferred to a multiwell plate and allowed to dry overnight at 37ºC, dissolved in 10µl loading buffer (0.2% w/v xylenecyanol and bromphenolblue in formamid) and denatured for 5min at 95ºC. The PCR products were separated by direct blotting electrophoresis (GATC 1500, MWG Biotech, Ebersberg, Germany) on a denaturing acrylamide gel (4% acrylamide/N,N'-methylenebisacrylamide 29:1 containing 6M urea in 0.9M Tris-0.9M Boric Acid-0.02M EDTA buffer) and simultaneously transferred to a Hybond N+membrane (Amersham). Signals were visualised by blocking the membrane in 1.5 w/v of blocking reagent in Buffer I, followed by incubation in diluted solution of anti-biotin alkaline phosphatase conjugate in Buffer I. The membrane was further washed three times with 1% Triton X-100 in Buffer I and equilibrated for 15min in assay buffer (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5). The membrane was covered for 5min with reaction buffer containing 10%v/v Sapphire II (Tropix) and 60µl CDPstar (Tropix) in 50ml assay buffer, followed by rinsing with a solution containing 1% v/v Sapphire II and 6µl CDPstar in 50ml assay buffer. Membranes were sealed in polysterol-foil and chemiluminescence was detected with Kodak X-o-mat X ray films. Exposition time varied from 10min to 45min depending on the efficiency of the PCR.

4.2.6) Parsimony Analysis

Family tree was constructed using parsimony analysis with PHYLIP 3.6 (Felsenstein 1989). To do parsimony analysis with PHYLIP 3.6 which accepts only binary discrete characters, the microsatellite allele size data were recoded as letters (a-e), in such a way that (a) is always the larger in size allele. Wagner parsimony analysis was done with the PARS program of

PHYLIP 3.6 (Felsenstein 1989). The Wagner parsimony method does not assume that the ancestral state is known or that mutation in one direction is more probable than the other.

For the analysis 91 out of the total of 105 genotyped loci were used, the program does not identify gaps and therefore all microsatellites with a heterozygous result for the pool of CF/1-C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and those with alleles missing were excluded from the analysis.

4.3) Results and Discussion

The aim of this study was to identify the genome wide strain distribution pattern of microsatellite alleles between the two inbred CF strains (CF/1- C*ftr*$^{TgH(neoim)Hgu}$, CF/3- C*ftr*$^{TgH(neoim)Hgu}$) and the three inbred strains (C57BL/6, BALB/c, DBA/2J) which were used for the generation of congenic strains.

4.3.1) Analysis of results

PCR amplification of microsatellites results in a number of 'shadow bands' both smaller and larger than the true alleles by integral numbers of repeat units. Slipped band mispairing is thought to be the major mechanism responsible for producing shadow bands at tandem dinucleotide repeats (Litt *et al*., 1993, Murray *et al*., 1993, Hauge *et al*., 1993). Titration of the number of PCR cycles and reduction of the primer concentration have been found to reduce the intensity of these extra bands and improve the quality of the PCR product (Todd *et al*., 1991).

Following direct blotting electrophoresis each allele is represented by a major band with strong intensity and by a variable number of shadow bands with reduced intensity when compared to the major band. Inbred strains are homozygous for all loci (isogenic), therefore each marker tested for these mice was expected to be homozygous. Informative microsatellite markers were chosen on the basis that they will present more than one allele between the three established inbred strains (C57BL/6J, BALB/c, DBA/2J) consequently three different genotypes were expected (following illustrating example). The alleles corresponding to the two inbred CF strains CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ were identified based on the alleles observed for the three inbred strains.

Example demonstrating interpretation of results

In order to genotype microsatellites, the repeat sequence is amplified via PCR, using primers that bind next to the repeat and include it into the PCR product (**NNNNN)**.

Allele c-Strain A

NNNNNNNNNNNNCACACACACACACACACACACANNNNNNN**NNNNN**

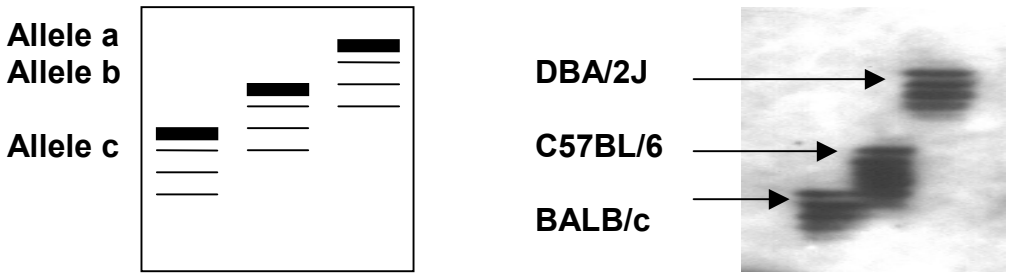
Allele b-Strain B

NNNNNNNNNNCACACACACACACCACACACACACACACACACANNNNNN**NNNN**

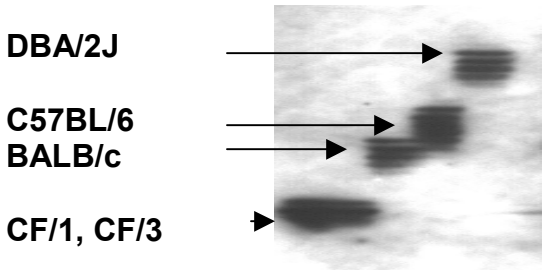
Allele a-Strain C

NNNNNNNNNCACAACACACACACACACACACACACACCACACACACACACACANNNNNN**NNNN**

Amplification of repetitive sequences can be prone to the appearance of by-products despite the amplification of specific primers (amplification slippage). Different alleles of a certain marker display characteristic by-band patterns with each marker giving a characteristic pattern of alleles. Inbred strains are homozygous for all loci (isogenic) therefore each marker tested for these mice was expected to be homozygous. (Marker D6Mit236)



The alleles corresponding to the two inbred CF strains CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ were analysed based on the allele pattern corresponding to the three inbred strains.



4.3.2) Strain distribution pattern between the two CF strains and the three wild type inbred strains

In total 105 microsatellite markers were typed for the two inbred CF strains and the three inbred wild type strains. The results are summarised in the table below.

Marker	Chr.	cM	CF/1	CF/3	DBA/2	C57BL/6	BALB/c
D1Mit211	**1**	**15**	b (146)	b (146)	b (146)	c (139)	a (149)
D1Mit303	**1**	**34.8**	**124/130**	**124/130**	**a** (124)	**a** (130)	a (130)
D1Mit136	**1**	**59.6**	a (108)	a (108)	b (104)	c (102)	a (108)
D1Mit450	**1**	**79**	a (118)	a (118)	b (106)	a (118)	a (118)
D2Mit372	**2**	**27.3**	b (119)	b (119)	b (119)	b (119)	a (127)
D2Mit37	**2**	**45**	c (146)	c (146)	a (182)	b (174)	c (146)
D2Mit525	**2**	**61.2**	a (127)	a (127)	b (117)	0	a (125)
D2Mit285	**2**	**86**	0	b (150)	d (138)	a (152)	c (148)
D3Mit130	**3**	**3.9**	a (140)	a (140)	**c** (121)	b (123)	**c** (121)
D3Mit96	**3**	**23.3**	b (221)	b (221)	**a** (225)	c (211)	**a** (225)
D3Mit156	**3**	**45.2**	c (254)	c (254)	a 266	d (252)	b (260)
D3Mit254	**3**	**64.1**	a (150)	a (150)	c (128)	a (150)	b (138)
D3Mit163	**3**	**87.6**	a (147)	a (147)	a (147)	0	b (146)
D4Mit55	**4**	**19.8**	a (184)	a (184)	c (164)	a (184)	b (180)
D4Mit116	**4**	**40**	c (136)	c (136)	a (152)	c (136)	b (144)
D4Mit169	**4**	**58**	b (103)	b (103)	b (103)	a (105)	**b** (103)
D4Mit310	**4**	**71**	c (117)	c (117)	b (121	c (117)	a (127)
D5Mit193	**5**	**1**	b (136)	b (136)	a (147)	b (136)	c (134)
D5Mit107	**5**	**26**	c (133)	c (133)	c (133)	b (136)	a (145)
D5Mit205	**5**	**45**	a (149)	a (149)	b (133)	**a** (149)	**a** (149)
D5Mit43	**5**	**83**	a (138)	a (138)	**b** (132)	**b** (132)	**b** (132)
D6Mit50	**6**	**3.3**	c (164)	c (164)	c (164)	a (170)	b (168)
D6Mit351	**6**	**20.4**	b (112)	b (112)	a (122)	b (112)	a (122)
D6Mit209	**6**	**32**	c (130)	c (130)	a (138)	b (134)	c (130)

D6Mit69	**6**	**35.15**	c (144)	c (144)	a (168)	b (164)	c (144)
D6Mit263	**6**	**37**	***b (144)***	***a (168)***	a (168)	b (144)	b (144)
D6Mit284	**6**	**37.50**	**136/142**	**136/142**	c (136)	a (145)	e (133)
D6Mit67	**6**	**41.50**	a (158)	a (158)	d (148)	c (152)	b (156)
D6Mit108	**6**	**48.10**	a (141)	a (141)	c (129)	b (131)	c (129)
D7Mit152	**7**	**1**	b (130)	b 130)	**d** (123)	c (127)	**a** (133)
D7Mit229	**7**	**23**	a (143)	a (143)	a (143)	b (139)	c (123)
D7Mit216	**7**	**44**	b (184)	b (184)	a (186)	c 180)	b (184)
D7Mit330	**7**	**57.5**	b (124)	b (124)	a (134)	b (124)	a (134)
D7Mit109	**7**	**66**	a (120)	a (120)	c (108)	**b** (118)	**b** (118)
D8Mit143	**8**	**8**	a (279)	a (279)	b (269)	a (279)	a (279)
D8Mit289	**8**	**11**	a (158)	a (158)	d (148)	c (152)	b (156)
D8Mit4	**8**	**14**	***d (157)***	***b (197)***	c (195)	d (157)	a (200)
D8Mit24	**8**	**18**	a (168)	a (168)	a (168)	b (160)	0
D8Mit204	**8**	**20**	a (150)	a (150)	a (150)	b (148)	a (150)
D8Mit65	**8**	**22.5**	a (280)	a (280)	b (234)	b(234)	a (280)
D8Mit102	**8**	**37**	c (117)	c (117)	b (118)	a (124)	c (117)
D8Mit318	**8**	**57**	b (128)	b (128)	d (118)	c (124)	a (132)
D8Mit326	**8**	**72**	b (125)	b (125)	a (127)	c (123)	b (125)
D9Mit250	**9**	**5**	a (127)	a (127)	b (123)	b (123)	b (123)
D9Mit191	**9**	**26**	a (153)	a (153)	**c** (99)	b (147)	**c** (99)
D9Mit270	**9**	**43**	c (140)	c (140)	b (144)	a (146)	c (140)
D9Mit137	**9**	**66**	b (131)	b (131)	a (145)	a (145)	b (131)
D10Mit246	**10**	**5**	c (180)	c (180)	**b** (182)	**a** (190)	**c** (180)
D10Mit250	**10**	**19**	b (124)	b (124)	a (125)	a (125)	b (124)
D10Mit251	**10**	**21**	***d (128)***	***e (120)***	c (138)	**a** (144)	b (140)
D10Mit107	**10**	**26**	***b (105)***	***a (107)***	**b** (105)	**b** (105)	a (107)
D10Mit36	**10**	**29**	c (138)	c (138)	a (148)	b (146)	c (138)
D10Mit61	**10**	**32**	a (148)	a (148)	a (148)	0	a (148)
D10Mit261	**10**	**47**	a (114)	a (114)	c (104)	b (110)	d (108)
D10Mit103	**10**	**70**	b (144)	b (144)	c (142)	d (140)	a (146)
D11Mit2	**11**	**2.4**	b (122)	b (122)	a (124)	b (122)	c (112)

D11Mit271	**11**	**21**	a (120)	a (120)	a (120)	a (120)	b (116)
D11Mit164	**11**	**32**	b (121)	b (121)	b (121)	a (123)	b (121)
D11Mit208	**11**	**33**	b (123)	b (123)	b (123)	a (129)	b (123)
D11Mit350	**11**	**34.45**	***b (96)***	***a (102)***	b (96)	a (102)	b (96)
D11Mit4	**11**	**37**	(244)	**(244/310)**	b (300)	c (246)	e (242)
D11Mit90	**11**	**42**	***c (150)***	***b (152)***	a (176)	c (150)	b (152)
D11Mit322	**11**	**44**	***c (107)***	***b (109)***	b (109)	a (113)	b (109)
D11Mit99	**11**	**59**	c (120)	**(120/123)**	**c** (120)	a (124)	**b** (123)
D11Mit49	**11**	**77**	b (156)	b (156)	b (156)	a (158)	a (158)
D12Mit37	**12**	**1**	a (140)	a (140)	a (140)	a (140)	b (118)
D12Mit153	**12**	**15**	a (160)	a (160)	b (158)	d (142)	d (156)
D12Mit159	**12**	**38**	a	a	a	a	a
D13Mit3	**13**	**10**	0	0	a (196)	c (159)	b (188)
D13Mit224	**13**	**37**	b (95)	b (95)	b (95)	a (117)	b (95)
D13Mit194	**13**	**49**	c (146)	c (146)	a (152)	b (150)	c (146)
D13Mit263	**13**	**71**	a (128)	a (128)	a (128)	a (128)	b (122)
D14Mit11	**14**	**1**	a (158)	a (158)	a (158)	**b** (152)	**b** (152)
D14NDS1	**14**	**2.5**	***b (196)***	***c (190)***	a (201)	d (182)	c (190)
D14Mit202	**14**	**7**	***a (146)***	***b (144)***	a (146)	a (146)	b (144)
D14Mit44	**14**	**10**	***a (150)***	***b (148)***	a (150)	a (150)	b (148)
D14Mit254	**14**	**12**	***a (122)***	***b (108)***	a (122)	a (122)	a (122)
D14Mit234	**14**	**22.5**	a (131)	a (131)	d (113)	b (127)	c (121)
D14Mit155	**14**	**25**	c (201)	c (201)	a (210)	c (201)	b (205)
D14Mit262	**14**	**28.4**	**(154/158)**	c (154)	c (154)	d (124)	b (156)
D14Mit237	**14**	**32.5**	***b (125)***	***a (134)***	b (125)	b (125)	b (125)
D14Mit239	**14**	**42.5**	c (125)	c (125)	a (131)	b (129)	c (125)
D14Mit106	**14**	**48**	***c (307)***	***b (309)***	c (307)	a (312)	c (307)
D14Mit166	**14**	**52**	**(128/144)**	a (144)	b (134)	a (144)	c (128)
D14Mit266	**14**	**60**	**(176/180)**	**(176/180)**	a (180)	c (148)	b (176)
D15Mit102	**15**	**6.7**	a (201)	a (201)	a (201)	a (201)	b (188)
D15Mit100	**15**	**21.1**	a (129)	a (129)	c (113)	d (111)	b (127)
D15Mit61	**15**	**26**	a (100)	a (100)	b (98)	a (100)	a (100)

D15Mit46	**15**	**27.5**	***c (142)***	***d (140)***	a (152)	b (150)	e (138)
D15Mit270	**15**	**28.4**	a (200)	a (200)	b (188)	a (200)	c (180)
D15Mit144	**15**	**32**	c (127)	c (127)	a (145)	c (127)	b (143)
D15Mit70	**15**	**47.7**	a (152)	a (152)	**b** (144)	a (152)	**b** (144)
D15Mit35	**15**	**61.7**	a (146)	a (146)	c (136)	b (142)	a (146)
D16Mit146	**16**	**16.9**	b (120)	b (120)	b (120)	**a** (123)	**a** (123)
D16Mit76	**16**	**43**	a (108)	a (108)	**a** (108)	b (89)	**a** (108)
D17Mit117	**17**	**29.4**	a (127)	a (127)	a (127)	b (123)	a (127)
D17Mit142	**17**	**47.4**	**(123/128)**	c (123)	**c** (123)	**a** (147)	c (123)
D18Mit18	**18**	**2**	b (142)	b (142)	**a** (144)	**a** (144)	c (140)
D18Mit110	**18**	**4**	***d (127)***	***a (153)***	a (153)	b (149)	c (133)
D18Mit21	**18**	**6**	***c (134)***	***b (136)***	c (134)	c (134)	a (140)
D18Mit34	**18**	**12**	c (128)	c (128)	a (138)	b (134)	c (128)
D18Mit54	**18**	**26**	c (144)	c (144)	c (144)	a (148)	b (146)
D18Mit8	**18**	**47**	a (77)	a (77)	a (77)	b (75)	c (71)
D19Mit68	**19**	**6**	a (136)	a (136)	b (132)	a (136)	c (122)
D19Mit40	**19**	**25**	a (112)	a (112)	b (106)	a (112)	c (102)

Table 2. Strain distribution pattern between the three inbred strains (C57BL/6, BALB/c, DBA/2J) and the two inbred CF lines (CF/1- C*ftr*$^{TgH(neoim)Hgu}$, CF/3- C*ftr*$^{TgH(neoim)Hgu}$) with 105 microsatellite markers. Letters represent alleles and allele size is in alphabetical order with **a** used for the longest allele. The size of the fragment in basepairs is also indicated in parenthesis. The thick black lines are used in order to separate the results between different chromosomes. Marker sizes indicated in the table with underlined italic bold (***i***) characters, are those found polymorphic between the two inbred CF strains (n=13). Those markers typed in order to refine the polymorphic loci between the two CF strains are indicated by grey boxes ( ). Six alleles represented by 0 did not amplify and for D12Mit159 allele size could not be assigned for any of the five alleles based on the given size on the database. For eight markers heterozygosity was documented (**in bold**) and for 19 markers the observed allele size did not agree with the allele size documented in the database for the same strains (indicated with **bold** letters in the table).

4.3.3) Analysis of the results for CF/1- C$ftr^{TgH(neoim)Hgu}$ and CF/3- C$ftr^{TgH(neoim)Hgu}$

4.3.3.1) Informative microsatellites between the two inbred CF strains.

Sixteen markers out of 105 microsatellites typed were polymorphic between the two CF strains (CF/1- C$ftr^{TgH(neoim)Hgu}$ and CF/3- C$ftr^{TgH(neoim)Hgu}$) indicating that these two strains are not identical to one another at all loci. This could be a direct impact of the inbreeding procedure which was used in order to generate these two strains. The outcross at the beginning of the inbreeding of the two CF strains was between outbred animals since the founder animal was generated using the outbred MF1 strain. During inbreeding random segregation and independent assortment will lead to animals in each generation that are genotypically distinct. When two siblings are chosen randomly to become the parents of the next generation there is the probability that they will be identically homozygous for a particular locus (0.25), homozygous for a different allele (0.25) or identically heterozygous (0.5). Depending on which animals in each generation are chosen for a subsequent breeding is as to which parental allele will be fixed i.e. become homozygous and will remain fixed as long as the brother x sister mating continues, except that mutations occur at this locus.

4.3.3.2) Heterozygosity

Residual heterozygosity was detected for eight microsatellites, between the two inbred CF strains (CF/1- C$ftr^{TgH(neoim)Hgu}$ and CF/3- C$ftr^{TgH(neoim)Hgu}$) indicating that the two strains were still segregating at F_{22+}. After 20 generations of brother x sister mating, a certain percentage of loci will remain heterozygous this is because linked loci do not assort independently, instead large chromosomal segments are inherited as units varying from one generation to the next. By 60 generations of inbreeding or higher mice can be considered homozygous with a probability of 99% in case there is no selection mutation (Silver 1995).

4.3.4) Stability of the inbred stains.

Fragment size of 19 microsatellite markers genotyped for the three inbred strains were different from the fragment size indicated in the database. This verifies the fact that there is a certain degree of genetic divergence between inbred strains maintained in different institutes. The reason for this observed difference may be due to mutations which had no visible effect on the phenotype and therefore remained undetected and became fixed in a homozygous state through consecutive inbreeding.

4.3.5) Comparison of strain distribution patterns.

4.3.5.1) Comparison of the three inbred strains (C57BL/6, BALB/c and DBA/2J).

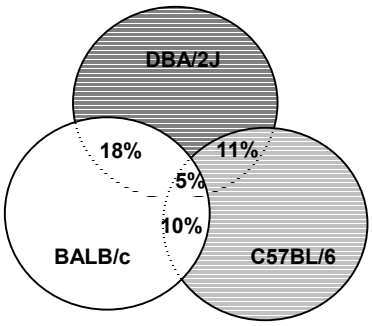


Figure 2. Comparison of the three inbred strains (C57BL/6, BALB/c and DBA/2J). From the total of 105 microsatellites typed 18% are shared between BALB/c and DBA/2J, 11% between DBA/2J and C57BL/6 and 10% between BALB/c and C57Bl/6. Only 5% are shared between all three inbred strains

Most inbred laboratory mouse strains are known to have originated from a mixed but rather limited population in a few laboratories (Silver 1995, Beck et al., 2000). Assembly of the murine genome sequence from the C57BL/6J strain (Boguski 2002)and alignment with sample sequence from other strains revealed long segments of either extremely high (40 SNPs per 10kd) or extremely low (0.5 SNPs per 10kb) polymorphism rates (Nadeau 2002). Only one third of the genome falls into long regions >1Mb of a high SNP rate suggesting that the genomes of the laboratory inbred strains are mosaics with the vast majority of segments deriving from *M.m. domesticus* and *M.m. musculus* (Wade *et al*., 2002).

Unlike the reported limited variation of Single Nucleotide Polymorphisms (SNPs) and consistent with the methodology used in the choice of microsatellite markers (three different

alleles between the three inbred strains) 59 out of the total 105 (56%) markers were polymorphic between the three inbred strains confirming previous findings of considerable allelic variation between microsatellites of different inbred strains of mice (Mc Pherson 1996)

4.3.5.2) Comparison of the two inbred CF strains and the three inbred wild type strains.

CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ show considerable allelic variation when compared with either one of the three inbred strains (Figure 3, Figure 4, Figure 5).

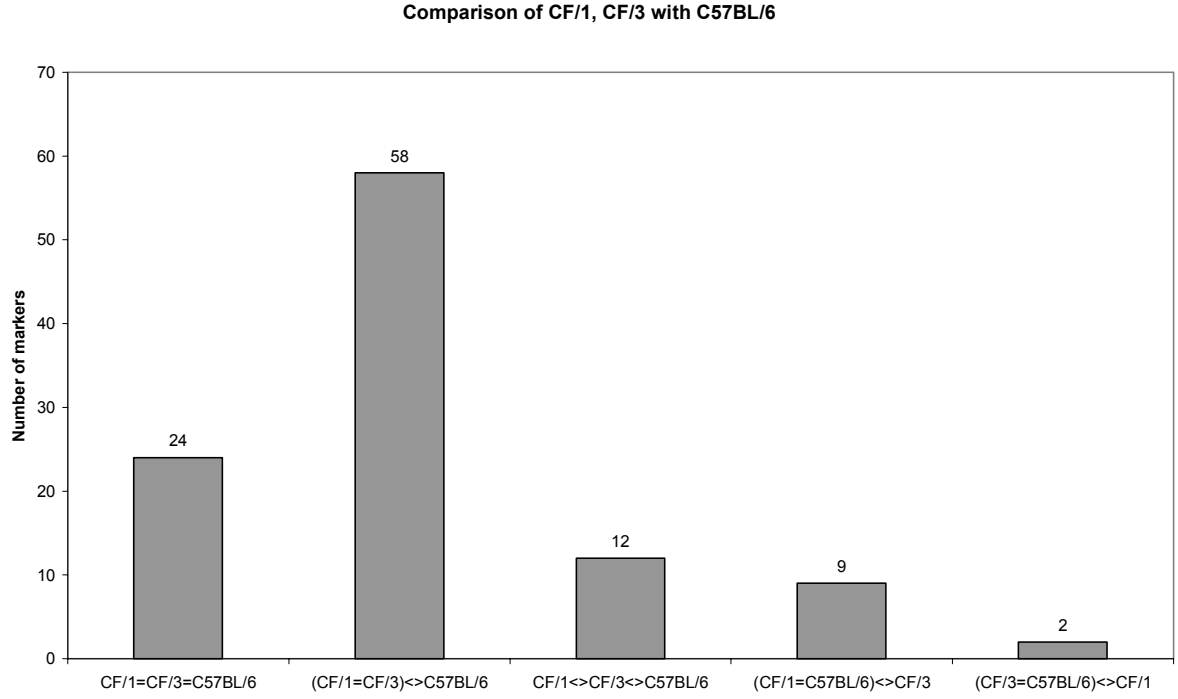


Figure 3. Comparison of microsatellite markers shared between CF/1, CF/3 and C57BL/6. (= same; <> different). 24/105 were shared between all three strains whereas the majority of the satellites 58/105 were shared between CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and were different with C57BL/6. 12/105 were polymorphic between all three strains. C57BL/6 shared 9/105 with CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and 2/105 with CF/3- C*ftr*$^{TgH(neoim)Hgu}$ from the polymorphic markers between the two inbred CF strains.

58/105 microsatellites were variable in size with the C57BL/6, 52/105 with the BALB/c and 58/105 with DBA/2J. This variation is comparable with the variation observed between the three inbred strains (58/105), indicating that the inbreeding procedure which gave rise to the two inbred CF strains resulted in mouse strains with a high degree of genetic variability when

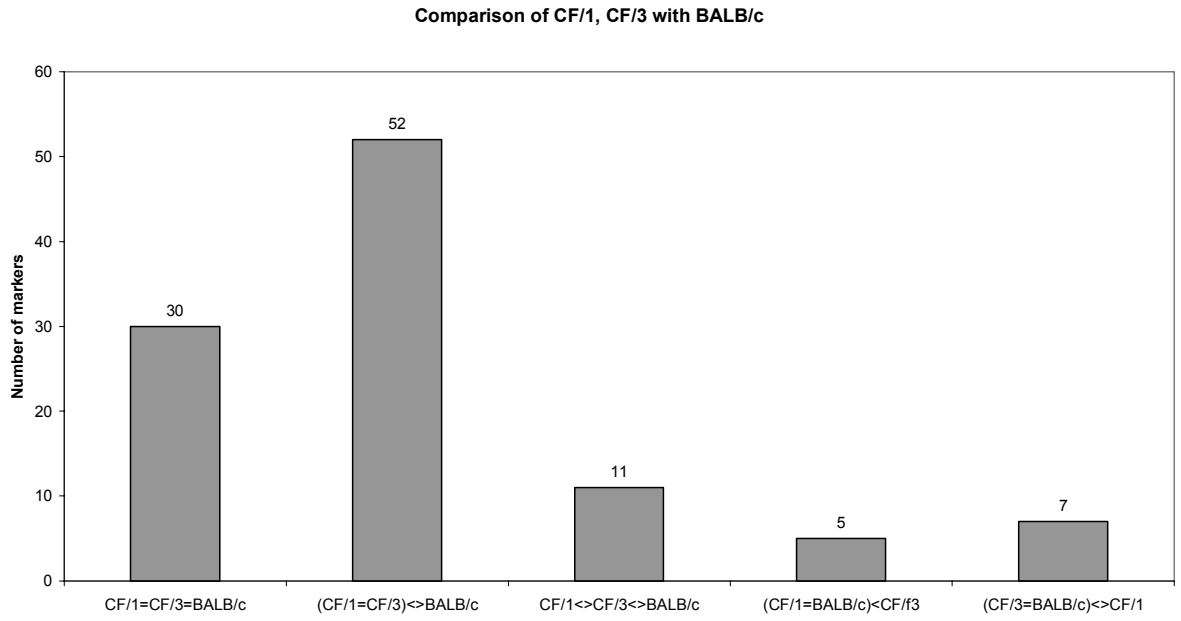


Figure 4. Comparison of microsatellite markers shared between CF/1- C*ftr*$^{TgH(neoim)Hgu}$, CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and BALB/c. (= same; <> different). 30/105 were shared between all three strains whereas the majority of the satellites 52/105 were shared between CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and were different with BALB/c.11/105 were polymorphic between all three strains. BALB/c shared 5/105 with CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and 7/105 with CF/3- C*ftr*$^{TgH(neoim)Hgu}$ from the polymorphic markers between the two inbred CF strains.

compared with the three established laboratory strains. CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ are the result of an inbreeding procedure at the beginning of which the outcross was between outbred animals since the founder animal was generated using the outbred MF1. The microsatellite allelic distribution of the MF1 mouse stock can not be investigated since in non isogenic mouse strains, mice are not identical with each other and an intrastrain allelic variability puts a restrain in the analysis of the resulting strain distribution pattern.

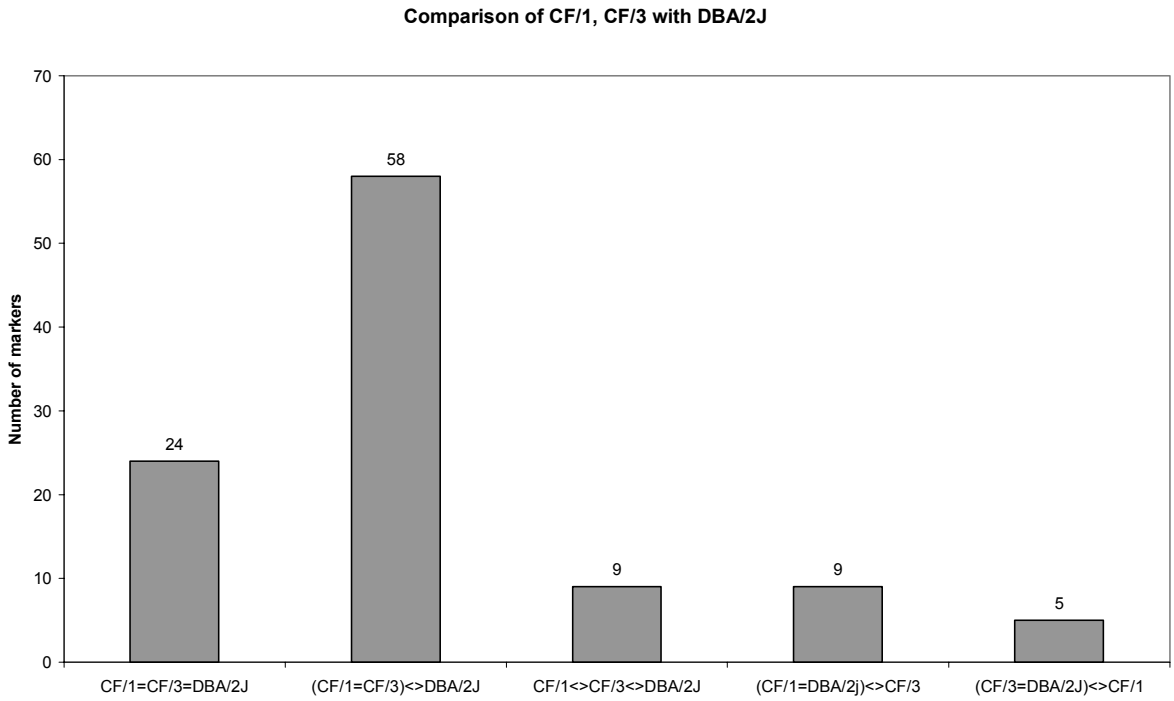


Figure 5. Comparison of microsatellite markers shared between CF/1- C*ftr*$^{TgH(neoim)Hgu}$, CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and DBA/2J. (= same; <> different). 24/105 were shared between all three strains whereas the majority of the satellites 58/105 were shared between CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ and were different with DBA/2J. 9/105 were polymorphic between all three strains. DBA/2J shared 9/105 with CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and 5/105 with CF/3- C*ftr*$^{TgH(neoim)Hgu}$ from the polymorphic markers between the two inbred CF strains.

One can only speculate that the considerable amount of genetic variation on the DNA level observed between the two CF strains and the inbred strains investigated in this study is the result of genetic difference between the outbred MF1 stock, and 129P2 (source of ES) with the three inbred strains.

Sequence variations between the laboratory strains as detected by restriction enzymes is difficult to find. The aim of this study was to identify the strain distribution patern between the two CF strains and further between the three inbred strains before the analysing the introgressed *Cftr*$^{TgH(neoim)Hgu}$ mutation in the three inbred backgrounds B6.129P2(CF/3)-*Cftr*$^{TgH(neoim)Hgu}$, C.129P2(CF/3)-*Cftr*$^{TgH(neoim)Hgu}$ and D2.129P2(CF/3)-*Cftr*$^{TgH(neoim)Hgu}$ animals.

4.3.6) Family tree by parsimony analysis

Although there are now many microsatellite markers available (primarily MIT markers from Dietrich *et al*., 1996) and the average density is about four markers/cM, it is quite often difficult to find sufficient polymorphic markers for a particular pair of inbred strains. To facilitate whole-genome scan experiments for the two inbred CF strains, CF/1- *Cftr*$^{TgH(neoim)Hgu}$ and CF/3- *Cftr*$^{TgH(neoim)Hgu}$, and the three inbred strains BALB/c, C57BL/6 and DBA/2J a panel of 105 microsatellite markers were selected for this study on the basis of spacing and polymorphism between the three inbred strains. CF/1- *Cftr*$^{TgH(neoim)Hgu}$ and CF/3- *Cftr*$^{TgH(neoim)Hgu}$ have not been characterised previously with any of these markers. In order to gain more information on the interrelationship of the two inbred CF strains and the three inbred strains the results obtained from the whole genome scan for all five strains were used for a parsimony analysis. Microsatellites offer a widely dispersed, selectively neutral set of characters that lends itself conceptually to parsimony analysis (Schalkwyk *et al*., 1999, Witmer *et al*., 2003,). Information on the interrelationships of strains is useful because of the importance of polymorphism in designing crosses and the background in assessing phenotypes. Atchley and Fitch (1993) performed a phylogenetic analysis employing classical genotypes from 144 gene loci in 24 inbred mouse strains, using parsimony analysis. Schalkwyk *et al*., (1999) performed a similar analysis using data from 128 SSLP loci in 11 inbred strains. Witmer *et al*., (2003) used the allele size data for 314 markers for 54 strains and constructed a mouse family tree using Wagner parsimony analysis.

Using the results of 91 of the 105 loci spread over the genome a phylogenetic tree was constructed representing the relationship of the two inbred CF strains and the three inbred strains. X and Y chromosomes were not considered in this analysis. Most new mutations in microsatellite markers (SSLP) are thought to result from gain or losses of a single repeat unit (Weber and Wong 1993, Amos *et al*., 1996, Primmer *et al*., 1998). Multiple repeat units can be added or deleted between alleles, and sometimes the entire repeat is duplicated. Parsimony

analysis considers specific mutation steps rather than the simple proportion of alleles that are the same size or different for a given marker. It assumes that species and characters evolve independently, it does not assume that the ancestral state is known or that mutation in one direction is more probable than the other. As in previous analyses without a defined model for the process of addition or deletion of multiple repeat units it was not clear how this process might affect the analysis. For the parsimony analysis in this study allele sizes were coded as letters (a-e) without assuming that a particular allele is ancestral. Wagner parsimony analysis using the PARS program of PHYLIP 3.6 (Felsenstein 1989), was performed. User options were selected to generate an unrooted tree because of a) low marker size b) no ancestral state for allele sizes was assumed for analysis. A consensus family tree is shown in Figure 6.

This tree has four groups of branches, labelled 1 to 4. Group 1 is comprised from the two inbred CF strains CF/1- *Cftr*$^{TgH(neoim)Hgu}$ and CF/3- *Cftr*$^{TgH(neoim)Hgu}$. Group 2 contains C57BL/6, group 3 contains DBA/2J and group 4 contains BALB/c.

For the three inbred strains C57BL/6, BALB/c and DBA/2J the tree derived is largely congruent with expectations from the known history of the strains and with the analyses of Atchley and Fitch (1993), Shalkwyk *et al*., (1999) and Witmer *et al*., (2003) indicating that the observed branching order is not the result of a subset of the recorded data which was collected for a different purpose. The two inbred CF strains CF/1- *Cftr*$^{TgH(neoim)Hgu}$ and CF/3 - *Cftr*$^{TgH(neoim)Hgu}$ which derived from the same founding pair form a strongly supported group not distant from each other but distant from the other three groups. These results are strongly supported by the microsatellite data but to make the picture more complete a larger number of markers would be necessary.

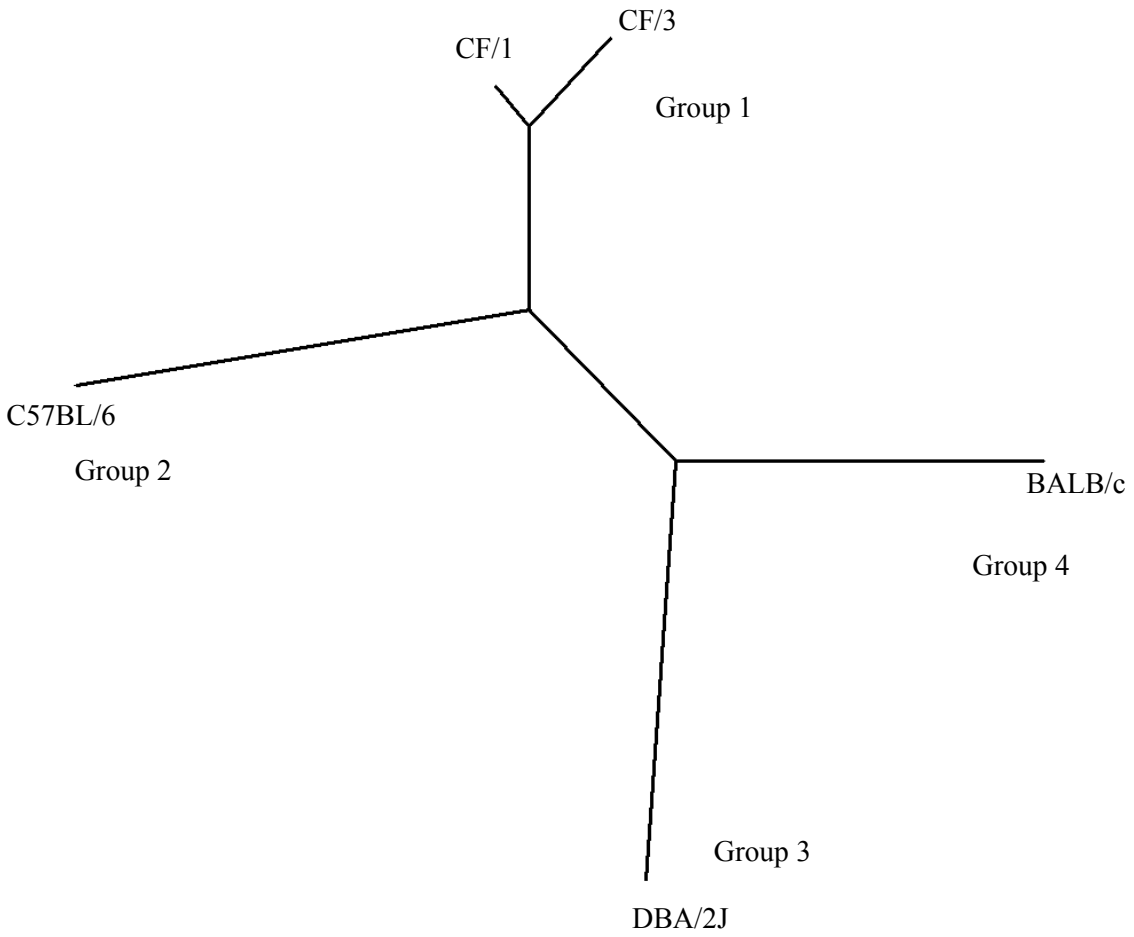


Figure 6. Family tree. Mouse strains are organised into 4 groups of branches. Group 1:CF/1 and CF/3; Group 2: C57BL/6; Group 3: DBA/2J; Group 4: BALB/c. The length and angles of the branches are optimised for printing and are not quantitative measures of evolutionary distance between strains.

4.5) Conclusions.

At present several models of CF have been described in the literature, however, one can not easily make comparisons on these animal models because of a) differences in the mutation induced to disrupt the murine *Cftr* gene on different genetically defined backgrounds; and b) different controlled environmental factors (diet, housing conditions). The ability to place (introgress) mutations on a different genetic background under the same housing conditions does not only help to bypass obstacles mentioned above but makes it possible to dissect the

role of the mutation induced, from the genetic background, and study the effect of modifier genes. However, before embarking in such backcrosses one has to pay attention on the genetic relationships of the strains that will be used for the generation of congenic animals and choose those which are not so closely related. In this study microsatellite genome scan of the two inbred CF strains and the three wild type standard strains revealed a high degree of difference between their genetic backgrounds, making the three inbred strains good candidates for the generation of congenic animals whereby genetic background based phenotypic differences can be studied.

Chapter 4-References

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Summary

- The laboratory mouse has played a key role in biomedical research. The development of mouse models for cystic fibrosis (CF) has provided the opportunity to dissect disease pathogenesis, correlate genotype and phenotype, study disease-modifying genes and develop novel therapeutics. Characterisation of the different CF mouse models has demonstrated the same primary phenotypes which included intestinal obstruction and characteristic intestinal and airway electrophysiology. Phenotypic variations between those various mouse models have been shown to relate either to the specific mutation, to different environmental influences and on independently segregating modifier genes. In *Chapter 1* an overview of these various mouse models and their phenotypes is given.
- In order to dissect the role of the mutation from the genetic background CF/3-C*ftr*$^{TgH(neoim)Hgu}$ mice served as donors for the development of the three congenic strains C57BL/6, BALB/c and DBA/2J, with selection for C*ftr*$^{TgH(neoim)Hgu}$ for 10 generations. Genotyping of the insertion mutation was conducted by Southern analysis of *Xba*I/*Sal*I restricted genomic DNA from spleen. In *Chapter 2* the introduction of an alternative genotyping technique utilising informative *Cftr* intragenic microsatellite markers in order to follow germline transmission of the mutated *Cftr* locus in the three inbred backgrounds was described. Utilisation of this "high-throughput" PCR based protocol provided a useful means whereby unidentified events such as recombination and vector excision could be monitored.
- In *Chapter 3* the phenotypic characterisation of the two inbred CF strains CF1-C*ftr*$^{TgH(neoim)Hgu}$ and CF3- C*ftr*$^{TgH(neoim)Hgu}$ is described. Quantification of the amounts of wild type Cftr protein produced by the two inbred CF strains was

carried out using western blot analysis and the CFTR-specific antibody R3195. The result indicated that both CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ produce reduced amounts of wild type protein. Overall the electrophysiological profile of the two stains was indistinguishable and argued for amelioration of the CF basic defect in these mice. Based on these phenotypic observations CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ mice provide excellent models for the identification of those genetic factors which influence disease phenotype and relate to those cellular processes which are involved in both the transcriptional and translational level influencing both mRNA and protein processing.

- In *Chapter 4* the genetic characterisation of the two inbred CF strains and the three inbred wild type standard inbred strains is described. A genome scan was performed for the five strains with 105 polymorphic DNA markers. The two inbred CF strains showed considerable allelic variation when compared with either one of the three inbred strains. This high degree of discordance (60%) between the genetic backgrounds of the strains used in this study may provide a useful tool for the interpretation of putative phenotypic differences which may account for the three congenic strains in the future. Hence, assisting in the identification of those factors which influence the phenotypic outcome observed for CF/1- C*ftr*$^{TgH(neoim)Hgu}$ and CF/3- C*ftr*$^{TgH(neoim)Hgu}$ in *Chapter 3*.

Appendix

1.1 Abbreviations

APS	Ammoniumperoxodisulfate
ATP	adenosine triphosphate
BALF	bronchoalveolar lavage fluid
BBMV	brush border membrane vesicles
CHO	Chinese hamster cells
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
DEPC	4,4´diisothiocyanatostilbene-2,2´-disulfonic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
ER	endoplasmic reticulum
LPS	lipopolysaccharide
MI	meconium ileus
MMR	mismatch repair
NBD	nucleotide binding domain
NER	nucleotide exchange repair
NMD	nonsense mediated decay
PA	*Pseudomonas aeruginosa*
PCR	Polymerase Chain Reaction
PKA	protein kinase A
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
SCC	short circuit current
SNP	Single Nucleotide Polymorphism
SPF	Specific pathogen free
TNF-α	tumour necrosis factor alfa

1.2 Material and equipment

1.2.1 Membrane, X-ray films

Hybond N^+ Nylon membrane	Amersham, Freiburg, D
KodakX-o-Mat AR X-ray films	Eastman Kodak, New Haven, CT, USA

1.2.2 Technical equipment

Autoclave	Tuttnauer, Breda, NL
Centrifuges:	
-Beckman GS-15R	Beckman Coulter, Fullerton, CA, USA
-Beckman Microfuge R	Beckman Coulter, Fullerton, CA, USA
-Eppendorf Mini Spin Plus	Eppendorf, Hamburg, D
Direct Blotter	
-GATC1500 direct blotting electrophoresis sequencer	GATC, Konstanz, D
Gel chambers for agarose electrophoresis	Forchungswerkstätten, MH Hannoner
Gel documentation system:	
-Gel-Print 2000	Biophotonics, Ann Arbor, MI, USA
Pippetes:	
-Mechanical	Eppendorf, Hamburg, D Gilson, Villier le Bel, F SLG, Gauting, D
- Multiwell	Biozym, Hess, Oldendorf, D Eppendorff, Hamburg, D
Photometer	Hitachi, Tokyo, J

Power supplies	BioRad, Hercules, CA, USA
Scales	Sartorius, Göttingen, D
Thermocycler	Hybaid, Teddington, UK
Thermomixer	Eppendorf, Hamburg, D
UV translluminator	Bachofer, Reutlinger, D

1.2.3 Consumables

Filter pipette tips (sterilile)	Greiner Bio-One, Frickenhausen, D
Latex exam Gloves (powder free)	Kimberly Clark, Zaventem, B
Multiwell PCR Plates	Greiner.Bio-One
Pipette tips	Eppendorff, hamburg, D
	Sarstedt, Nümbrecht, D
Reaction vials	Eppendorff, Hamburg, D
	Sarstedt, Nümbrecht, D

1.2.4 Chemicals

1.2.4.1 General Chemicals

Acrylamide:	
-AccuGel 19:1	National Diagnostics Atlanda, Georgia, USA
-Rotiphorese Gel 40	Roth, Karlsruhe, D
Agarose ultra pure	Life Technologies, Paisley, Scotland, UK
Ammoniumperoxodisulfate (APS)	Roth, Karlsruhe, D
Boric acid, crystalline (H3BO3)	Merck, Darmstadt, D
Bromphenolblue	Serva, Heidelberg, D
Diethyl-pyrocarbonate (DEPC)	Sigma-Aldrich, Steinheim, D
Chloroform p.a	Roth, Karlsruhe,D
Acetic Acid, 96%	Merck, Darmstadt, D
Ethanol	JT Baker, Deventer, NL
Formamide	Sigma-Aldrich, Steinheim, D
Glycerol	Fluka, Buch, CH

Isomylalcohol	Roth, Karlsruhe,D
Isopropanol	Fluka, Buch, CH
KCl, p.a	Merck, Darmstadt, D
MgCl2 x 6 H2O, p.a	Merck, Darmstadt, D
NaCl, p.a	Merck, Darmstadt, D
NaOH, p.a	Merck, Darmstadt, D
Na2-EDTA	Merck, Darmstadt, D
Paraffin	Fluka, Buch, CH
Phenol:	
-Roti-Phenol pH 7.5-8.0	Roth, Karlsruhe
TEMED	Serva, Heidelberg, D
Tris ultra pure	ICN, Aurora, Ohio, USA
Triton x 100	Serva, Heidelberg, D
Urea, pearls, purest	Merck, Darmstadt, D
Xylenecyanol	Sigma, St.Louis, MO, USA

1.2.4.2 Buffers and standard solutions

Developing Buffer I:

100mM Tris-HCl pH 7.5
150mM NaCl

Developing Buffer III:

100mM Tris-HCl pH 9.5
100mM NaCl
50mM $MgCl_2$

Loading buffer for agarose gels:
0.05% (w/v) xylenecyanol, 0.05% (w/v) bromphenolblue, 40% (w/v) glycerol

Loading buffer for direct dlotter:
0.2% (w/v) xylencyanol, 0.2% (w/v) bromphenolblue in formamide

Lysis buffer:

50mM	Tris-HCl	pH 7.5
109.5 g/l	saccharose	
1% (w/v)	Triton X-100	

STE:

50mM Tris-HCl pH 7.5
100mM NaCl
1mM Na_2-EDTA

10 x TBE:

1.275M Tris-HCl pH 9.0
0.42M H3BO3
0.024M Na2-EDTA

TE:

10mM Tris-HCl pH 8.0
1 mM Na2-EDTA

1.2.4.3 Biochemicals, enzymes, enzyme-related buffers and photo-chemicals.

Anti-Biotin-AP, Fab-fragments	Roche Diagnostics, Mannheim, D
Blocking Reagent	Roche Diagnostics, Mannheim, D
CDP star	Tropix, Bedford, MA, USA
Developing solution for X-ray films	Agfa-Gevaert, Mortsel, B
Desoxynucleotides (dNTPs)	Roth, Karlsruhe, D
Express fixative salt	Tetenal, Norderstedt, D
Luminescence-enchancer Sapphire II	Tropix, Bedford, MA, USA
Oligonucleotides	Invitrogen, Life technologies, Paisley, UK
Restriction enzymes	New England Biolabs, Frankfurt/Main, D
Taq polymerase	InviTek, Berlin, D

dNTPs:

100 mg dNTP (A,G,C or T)
+100 µl Tris-HCl 1M pH 7.0
+900 µl H_2O_{STE}
add 1.1 ml H_2O_{STE}

2 µM of each dNTP was added together and filled up to 1ml with H_2O_{STE} resulting in a dNTP premix solution with a final concentration of 2mM of each dNTP.

PCR 10 x NH_4^+ reaction buffer (InviTek).

500 mM Tris-HCl pH 8.8
160 mM $(NH_4)_2SO_4$
0.1% Tween 20

1.3) List of figures and tables

Chapter 2.

Chapter 3

Curriculum Vitae

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Education

1999-2000 University of Glasgow (MMSc, Medical Genetics), UK
1995-1998 University of Hull (BSc, Molecular Biology and Biotechnology), UK
1991-1994 2nd Lyceum of Grevena, Greece
1988-1991 2nd Gymnasium of Grevena, Greece
1981-1988 2nd Elementary School of Grevena, Greece

Educational Qualifications

MSc. Medical Genetics. September 2000

BSc. Molecular Biology and Biotechnology, (Special Honours). July 1998

Apolytirio (Graduation Certificate). June 1994

Qualifications in English Language

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English for Academic Purposes (University of Hull Language Institute), June 1997

List of publications

1) Charizopoulou N, Jansen S, Dorsch M, Stanke F, Dorin JR, Hedrich HJ, Tümmler B. **Instability of the insertional mutation in CftrTgH(neoim)Hgu cystic fibrosis mouse model.** *BMC Genet. 2004 Apr 21*