Research article

Instability of the insertional mutation in Cftr<sup>TgH(neoim)Hgu</sup> cystic fibrosis mouse model

Nikoletta Charizopoulou*1, Silke Jansen1, Martina Dorsch2, Frauke Stanke1, Julia R Dorin3, Hans-Jürgen Hedrich2 and Burkhard Tümmler1

Address: 1Klinische Forschergruppe, OE 6710, Medizinische Hochschule Hannover, D-30625 Hannover, Germany, 2Zentrales Tierlaboratorium, OE 8600, Medizinische Hochschule Hannover, D-30625 Hannover, Germany and 3MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, UK

Email: Nikoletta Charizopoulou* - charizopoulou.nikoletta@mh-hannover.de; Silke Jansen - jansen.silke@mh-hannover.de; Martina Dorsch - Dorsch.martina@mh-hannover.de; Frauke Stanke - Stanke.frauke@mh-hannover.de; Julia R Dorin - julia@hgu.mrc.ac.uk; Hans-Jürgen Hedrich - Hedrich.hans@mh-hannover.de; Burkhard Tümmler - Tuemmler.burkhard@mh-hannover.de

* Corresponding author

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Abstract

Background: 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. Moreover, the introduction of the same mutation on different inbred strains generating congenic strains facilitated the search for modifier genes. From the original Cftr<sup>TgH(neoim)Hgu</sup> CF mouse model we have generated using strict brother × sister mating two inbred Cftr<sup>TgH(neoim)Hgu</sup> mouse lines (CF/1 and CF/3). Thereafter, the insertional mutation was introgressed from CF/3 into three inbred backgrounds (C57BL/6, BALB/c, DBA/2J) generating congenic animals. In every backcross cycle germline transmission of the insertional mutation was monitored by direct probing the insertion via Southern RFLP. In order to bypass this time consuming procedure we devised an alternative PCR based protocol whereby mouse strains are differentiated at the Cftr locus by Cftr intragenic microsatellite genotypes that are tightly linked to the disrupted locus.

Results: Using this method we were able to identify animals carrying the insertional mutation based upon the differential haplotypic backgrounds of the three inbred strains and the mutant Cftr<sup>TgH(neoim)Hgu</sup> at the Cftr locus. Moreover, this method facilitated the identification of the precise vector excision from the disrupted Cftr locus in two out of 57 typed animals. 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.

Conclusions: We present intragenic microsatellite markers as a tool for fast and efficient identification of the introgressed locus of interest in the recipient strain during congenic animal breeding. Moreover, the same genotyping method allowed the identification of a vector excision event, posing questions on the stability of insertional mutations in mice.

Background

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 [1] several
mouse models have been created. These fall broadly into two different categories; those designed to mimic clinical human mutations such as the F508del [2-4], G551D [5] and G480C [6], and those with a disrupted Cftfr gene resulting in either no or reduced production of CFTFR. 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 Cftfr knockout mice demonstrated differential severity of airway [7] and intestinal [8] disease. Candidate modulators for growth, airway and intestinal disease have been mapped to loci on chromosomes 1, 6, 7, 10 and 13 [9]; 1, 2, 10 and 17 [10]; 3 and 5 [11], respectively.

Dorin et al. [12] established a CF mutant mouse CfrftrTgH(neom)Hgu, using an insertional gene targeting vector to disrupt exon 10 of the Cfrftr 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 Cfrftr mRNA are produced as a result of exon skipping and aberrant splicing [13], but these mutant mice nevertheless displayed the electrophysiological defect in the gastrointestinal and respiratory tract which is characteristic of CF [14]. We have generated two different inbred lines named CF/1-CfrftrTgH(neom)Hgu and CF/3-CfrftrTgH(neom)Hgu using brother-sister mating for more that 26 generations. In order to test whether the genetic background of the CfrftrTgH(neom)Hgu mouse influences the development of the phenotype, we introgressed the mutation from the CF/3-CfrftrTgH(neom)Hgu into three different inbred strains (C57BL/6, BALB/c, DBA/2J) generating B6.129P2(CF/3)- CfrftrTgH(neom)Hgu, C.129P2(CF/3)-CfrftrTgH(neom)Hgu, D2.129P2(CF/3)-CfrftrTgH(neom)Hgu congenic mice. During backcrossing the targeted mutation was determined by Southern RFLP analysis of XbaI/SalI genomic digests with probe 1.2H (Figure 1) as outlined in the original report [12]. Here we describe an alternative genotyping technique utilising informative Cfrftr intragenic microsatellite markers in order to follow germline transmission of the mutated Cfrftr locus in the three inbred backgrounds. The four markers spanning 101 kb of the Cfrftr gene allowed straight forward differentiation between the two inbred CF strains and the three inbred wild type strains by microsatellite haplotype. Southern and microsatellite mutation genotypes were confirmed in 55 of 57 typed mice. 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 7.3 kb insertion vector had been excised from the Cfrftr locus. This spontaneous reversion to wild type sheds serious doubts for the stability of insertion mutations in heterozygous mice.

**Results**

From the original CfrftrTgH(neom)Hgu mutant mouse generated using insertional mutagenesis in the Cfrftr exon 10 [12] we have established two inbred CF strains CF/1-CfrftrTgH(neom)Hgu and CF/3- CfrftrTgH(neom)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 Cfrftr locus by intragenic microsatellite genotypes tightly linked with the intron 9 and exon 10 of Cfrftr chosen for insertion mutagenesis in the CfrftrTgH(neom)Hgu mouse mutant.

**Allele distribution between the strains. Consistent genotyping**

Four of the six tested Cfrftr 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 strains CF/1-CfrftrTgH(neom)Hgu and CF/3- CfrftrTgH(neom)Hgu (Figure 2). The two inbred CF/1-CfrftrTgH(neom)Hgu and CF/3- CfrftrTgH(neom)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) 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).

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 Cfrftr locus, we tested all three congenic strains. Fifty-seven animals (C.129P2(CF/3)-CfrftrTgH(neom)Hgu n = 31, B6.129P2(CF/3)- CfrftrTgH(neom)Hgu n = 9 and D2.129P2(CF/3)-CfrftrTgH(neom)Hgu n = 17) selected at random at generation N10F2 and N10F3 from both heterozygous and homozygous CF matings were compared in the 1.2H probe restriction XbaI/SalI RFLP and marker genotypes of the three informative intragenic Cfrftr microsatel-
Figure 1
Generation of the Cftr\textsuperscript{Tg\textsuperscript{H(neoim)Hgu}} mouse model. 

a) Insertional disruption of the murine Cftr gene and predicted gene structure as described by Dorin et al. (1992). Abbreviations: S, SalI; H, HindIII; X, XbaI. Map position of the CFneo1 and CFneo2 products is also indicated. b) Genotype analysis of heterozygous cf/+ matings. The probe 1.2H issued in order to identify via Southern hybridisation the congenic mice which carry the insertion. DNAs were digested with XbaI+SalI and probed with 1.2H. The upper hybridising fragment of 6.6 Kb represents the wild type allele, the lower 5 Kb fragment is diagnostic for the insertional mutation.
Figure 2  
**Microsatellite genotyping.**  
*a*) Localisation of the intragenic polymorphisms on the physical map of the murine *Cftr* gene.  
*b*) Analysis of the four informative intragenic microsatellites by direct blotting electrophoresis.  
*c*) Microsatellite alleles were ascertained by arbitrary 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.
lites (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 1).

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 2). In detail, mouse A was classified heterozygous CF by Southern and homozygous CF by the microsatellites and mouse B 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 1). 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 SalI site. The results of the PCR assays were consistent with the Southern data i.e. for mouse A both insert specific products were present indicating an intact vector on one chromosome, whereas for mouse B both products were absent. This data strongly suggests that the pIV3.5H insertion vector had been excised from the CF/3- CftrTgH(neoin)Hgu Cftr locus at least in mouse B.

| mouse | 1.2H | D6NC3 | D6Mit236 | D6NC5 |
|-------|------|-------|----------|-------|
| A     | cf/n | cf/cf | (20/20)  | cf/cf (20/20) |
| B     | n/n  | cf/cf | (20/20)  | cf/cf (20/20) |

Table 3: Primer sequences used for the amplification of the long range products.

| Primer name | Primer sequence 5’-3’ | Expected size | Allele |
|-------------|-----------------------|---------------|--------|
| Cfr-5012    | CCT TCC ATG TAC CCC TCC TCA CTX | 5012 bp | Wild type |
|             | CCC GGC ATA ATC CAA GAA AAT TG  |               |        |
| Cfr-5198    | TGT GGG AAA TCC TGT GTG GAA A | 5198 bp | mutant  |
|             | CTT CGT CGT ATG TTT TTT T    |               |        |
| Cfr-3736    | CAC ACA ACA TAC GAG CCG GAA G | 3736 bp | mutant  |
|             | TTT ATT GCC GAT CCC CTC AGA A |               |        |
| Cfr-3473    | CTC GTG CTT TAC GGT ATC GGC | 3473 bp | mutant  |
|             | TGC TGT AGT TGG CAA GCT TTG A |               |        |
Figure 3
**Long range PCR.** a) Map position of the long range PCR product corresponding to the 5012 bp 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.
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 5012 bp corresponding to wild type \textit{Cftr} allele and three primer sets corresponding to the mutant allele with the inserted sequence (Figure 3). Mouse B was positive only for the 5012 bp product confirming the absence of the pIV3.5H vector on both chromosomes, whereas mouse A was positive for all four products indicative of a CF heterozygous mouse. In mouse B the inserted vector had been excised from both chromosomes, and in mouse A in one chromosome.

**Primer walking**

The sequence integrity of the complete homologous targeted region was checked by primer walking. Fifteen sets of primers were designed from \textit{Cftr} exon 9 to intron 10 (Table 4, Figure 4), 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 \textit{Cftr} intron 9 where the vector was introduced via homologous recombination [12] the sequence was found to be 100% wild type \textit{Cftr} with no insertional vector (pIV3.5H) sequence retention. Small sequence alterations were observed when compared to the AF162137 database C57BL/6 derived sequence, most likely representing SNPs between the mouse strains used for the generation of the \textit{CftrTgH(neom)Hgu} mouse model (MF/1, 129P2) and the C57BL/6 mouse strain. In summary, since sequencing by primer walking revealed neither any loss of wild type \textit{Cftr} sequence nor retention of vector sequence, we conclude that in the two mice the pIV3.5H insertion vector had been completely removed (by the base) from the disrupted \textit{Cftr} locus.

| Primer name   | Location    | Sequence 5'-3' | Product Size |
|---------------|-------------|----------------|--------------|
| NCEx919-A     | 197720-197742 | TTT GGG GAA TTA CTG GAG AAA G | 419 bp      |
| NCEx919-B     | 198138-198117 | AGC TCG CTG ATA GGT TAT CCA | 400 bp      |
| NC10-A        | 198002-198023 | CCC CTC CTC ACT TCC ATT AAA | 649 bp      |
| NC10-B        | 198402-198381 | TTT AAG GCT CAG GGC TAA TTG | 550 bp      |
| NC11-A        | 198376-198396 | TTC CAC AAT TAG CCC TGA GC | 550 bp      |
| NC11-B        | 199024-199001 | TGA AGG AAA TTA CTG AAG CA | 563 bp      |
| NC12-A        | 199001-199024 | TGC TTT ACT AAT GAT TTT CTT CA | 575 bp      |
| NC12-B        | 199551-199531 | TAT GGA TCC CCA CAG CAG GT | 582 bp      |
| NC13-A        | 199394-199414 | CTC AGG GAT TTT CAC GGT TT | 582 bp      |
| NC13-B        | 199966-199946 | GCT TGG ATC TCT GGG AGC AC | 587 bp      |
| NC14-A        | 199741-199763 | GAT CAC AGG AGC CTA GCA TAG A | 575 bp      |
| NC14-B        | 200290-200268 | TTC ACT TTA CAA CCT GGC TTC A | 582 bp      |
| NC15-A        | 200122-200142 | ACT AGG AGA GGA TGC AAA AA | 480 bp      |
| NC15-B        | 200696-200676 | CCC AGT GTG AGA AGA TGC AC | 487 bp      |
| NC16-A        | 200572-200592 | TGC TCC CAG AAA TCT TCA CC | 413 bp      |
| NC16-B        | 201153-201133 | AGT TGT CAG AAG GAA ACC CA | 403 bp      |
| NC17-A        | 201134-201154 | TGG GTT CCC TCT TGA CAA CT | 403 bp      |
| NC17-B        | 201715-201695 | TTA GGT CCC CGT GCT TAC AC | 403 bp      |
| NC19-A        | 201739-201759 | TAG GTG GAT CCA TAA CCC CA | 403 bp      |
| NC19-B        | 202198-202199 | GGA CAG AGA AGC AGT GG | 403 bp      |
| NC20-A        | 202199-202219 | CCA CTC CTG CTT CTC TGT CC | 403 bp      |
| NC20-B        | 202668-202666 | AAA GAA GAG CGA GCC CCT AC | 403 bp      |
| NC21-A        | 202986-203006 | TTC TCT GAT TAT GCC GGG TA | 403 bp      |
| NC21-B        | 203387-203367 | TTT CCA GTG GGG GTG ACA CT | 403 bp      |
| NC22-A        | 203296-203316 | GGG CTT CAA GGG CTA ATT CT | 403 bp      |
| NC22-B        | 203775-203755 | ATG TGA TCC AGA CTG GCC TA | 403 bp      |
| NC23-A        | 203654-203674 | ATG CAT GGG GTG TGG TAC TT | 403 bp      |
| NC23-B        | 204277-204255 | TCC AAT GAT CTA CCT GTG TCC A | 403 bp      |
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 CftrTgH(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 [12]. 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- CftrTgH(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 [15]. Although it remains to be proven whether intragenic changes can account 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 background 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.

Excision

In two separate cases (mouse A and mouse B) the Southern insertional mutation genotype could not be verified with the three intragenic microsatellites. A heterozygous mouse A and a homozygous wild type mouse B, as indicated via Southern blot hybridisation were homozygous for the intragenic microsatellite genotype linked to the disrupted Cftr locus (CF/3- CftrTgH(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 B and in one chromosome in mouse A. Primer walking revealed that the 7.3 kb vector has been excised precisely from the mutated Cftr locus without causing any sequence alteration in the Cftr gene. Both mouse A and mouse B are littermates of the same two parental animals heterozygous for the mutation as indicated by Southern blot and Cftr intragenic microsatellite genotyping. Further investiga-

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Figure 4

Primer walking. Straight lines represent the fragment amplified by each primer set, overlapping sequences are represented by boxes.
tion on the remaining offspring (n = 7) (Table 5) of the same litter from these two parental animals revealed that three further animals had inconsistent genotyping with two animals (C and D) resembling littermate A and mouse E resembling littermate B, whereas four animals (F, G, H, I) were homozygous CF. The above supports the hypothesis that the excision event had occurred during gametogenesis in both the male and the female parental germlines.

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 [16-18]. 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 [19,20], 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 [21] 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 [22] 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.

**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 [23,24]. 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.

**Methods**

**Experimental animals**

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

**Generation of inbred Cftr<sup>TgH(neoim)Hgu</sup> mutant mice**

For the establishment of the inbred CF/1-Cftr<sup>TgH(neoim)Hgu</sup> and CF/3- Cftr<sup>TgH(neoim)Hgu</sup> 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 Cftr<sup>TgH(neoim)Hgu</sup> lines CF/1- Cftr<sup>TgH(neoim)Hgu</sup> and CF/3- Cftr<sup>TgH(neoim)Hgu</sup> which were generated by brother-sister mating for now more than 26 generations.

**Generation of congenic Cftr<sup>TgH(neoim)Hgu</sup> mutant mice**

CF/3- Cftr<sup>TgH(neoim)Hgu</sup> mice served as donors for the development of the three congenic strains C57Bl/6, BALB/c and DBA/2J, with selection for Cftr<sup>TgH(neoim)Hgu</sup> for 10 generations. Genotyping of the insertion mutation was conducted by Southern analysis of Xbal/Sall restricted genomic DNA from spleen [12].

**DNA purification**

High molecular weight DNA was isolated from 0.15 g spleen tissue, either fresh or thawed on ice after storage at -20 °C based on the protocol by Gross-Bellard et al. [25].

**Southern blot genotyping**

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

**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 [http://frodo.wi.mit.edu](http://frodo.wi.mit.edu) are listed in Table 6.

**Genotyping of microsatellites**

Microsatellite markers were genotyped in 96 well plates purchased from Greiner, Frickenhausen, pre-coated with 50 ng 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 30 µl, without oil overlay, using InvITaq polymerase (InvITek, Berlin). After PCR an 8 µl aliquot was transferred to a multiwell plate and allowed to dry overnight at 37 °C, dissolved in 10 µl loading buffer (0.2% w/v xylenecyanol and bromphenolblue in formamide) and denatured for 5 min at 95 °C. 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 6 M urea in 0.9 M Tris-0.9 M boric acid-0.02 M 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 (100 mM Tris-HCl, 150 mM 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 15 min in assay buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5). The membrane was covered for 5 min with reaction buffer containing 10% (v/v) Saphhire II (Tropix) and 60 µl CDPstar (Tropix) in 50 ml assay buffer, followed by rinsing with a solution containing 1% (v/v) Saphhire II and 60 µl CDPstar in 50 ml assay buffer. Signals were exposed to Kodak XR-R films and the exposition time varied from 10 min to 45 min. Evaluation of results was performed as described by Mekus et al. [26].

**Long-range PCR**

150 ng of DNA template was each amplified in 12 different premixes using the Failsafe™ PCR System (EPICEN...
PCR reactions were carried out in a total volume of 30 µl with InViTaq polymerase (InViTek, Berlin) in 96 well plates. Full-length and Sall restricted PCR products were separated by 2.5% agarose gel electrophoresis.

Table 7: Primer sequences used for the amplification of the Cftr intron 9-pMC1 vector plasmid sequence (CFneo2) and the neomycin-Cftr intron 9 (CFneo1) products.

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

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 [http://frodo.wi.mit.edu](http://frodo.wi.mit.edu). PCR reactions were performed on DNA with inconsistent Southern and microsatellite insertion mutation genotypes and controls in 96 well plates precoated with 50 ng of DNA template using InViTaq polymerase (InViTek, Berlin). Full-length products were separated on 2.5% agarose gels and visualised under UV illumination.

Sequencing

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

Authors’ contributions

NC devised the typing protocol by microsatellites and executed all microsatellite genotyping. She performed all experiments to unravel the nature of inconsistent Southern and microsatellite genotypes. SJ carried out the DNA extractions and the Southern blot analysis. MD participated in the supervision of the animal breeding and was responsible for the tissue collection. FS participated in microsatellite marker selection and assisted with the interpretation of the results. JRD provided us with the mouse model. HJH designed and supervised all animal breeding. BT conceived the study and participated in the design of experiments and result analysis. All authors contributed to the writing of this manuscript.

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