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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*$^{TgH(neoim)Hgu}$ CF mouse model we have generated using strict brother × sister mating two inbred *Cftr*$^{TgH(neoim)Hgu}$ 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*$^{TgH(neoim)Hgu}$ 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 *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 [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 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 [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- 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 [12]. 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 101 kb of the *Cftr* 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 *Cftr* locus. This spontaneous reversion to wild type sheds serious doubts for the stability of insertion mutations in heterozygous mice.

Results

From the original C*ftr*$^{TgH(neoim)Hgu}$ mutant mouse generated using insertional mutagenesis in the *Cftr* exon 10 [12] 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. 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.

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 strains 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).

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) selected at random at generation $N_{10}F_2$ and $N_{10}F_3$ from both heterozygous and homozygous CF matings were compared in the 1.2H probe restriction *Xba*I/*Sal*I RFLP and marker genotypes of the three informative intragenic *Cftr* microsatel-

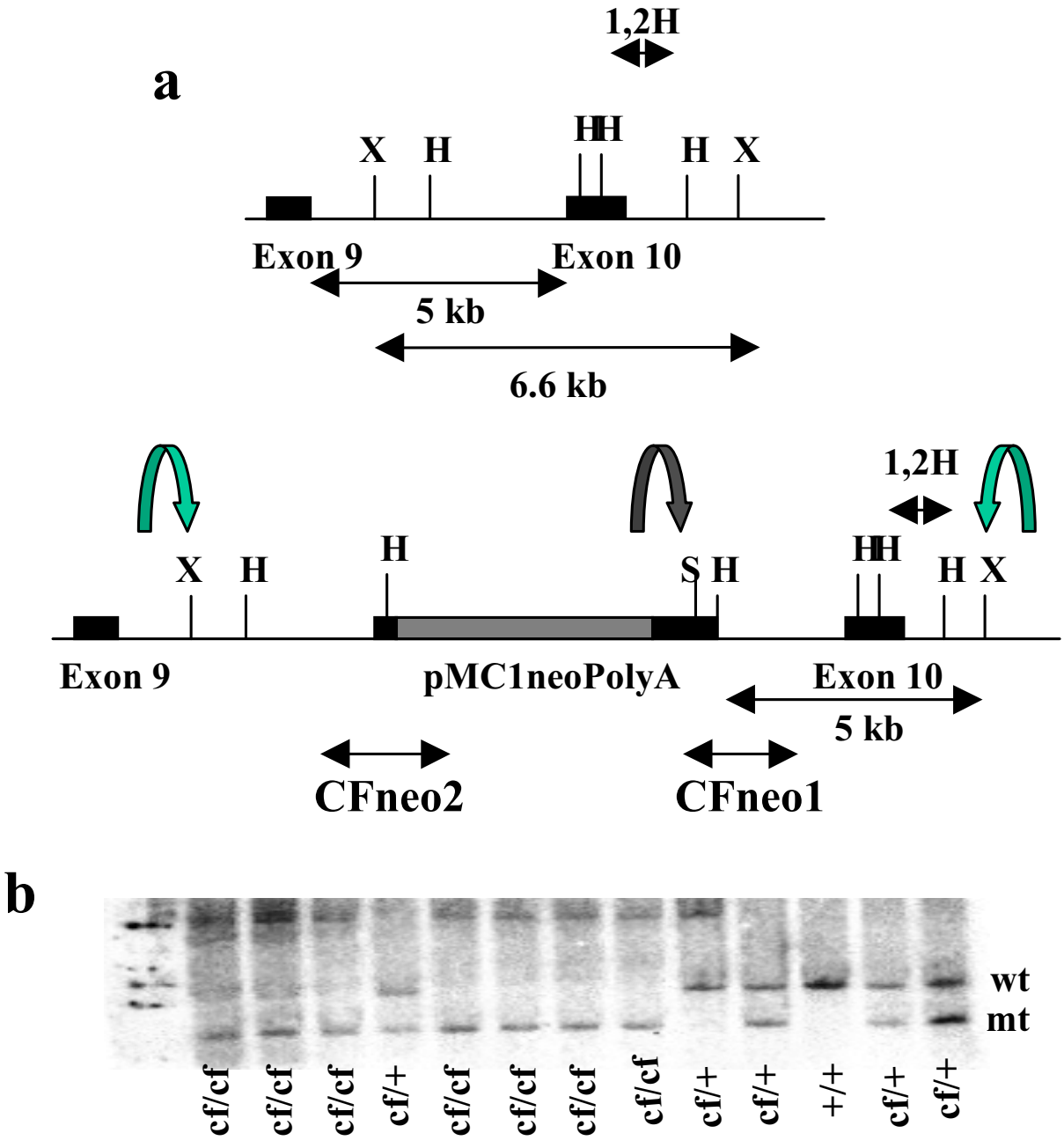
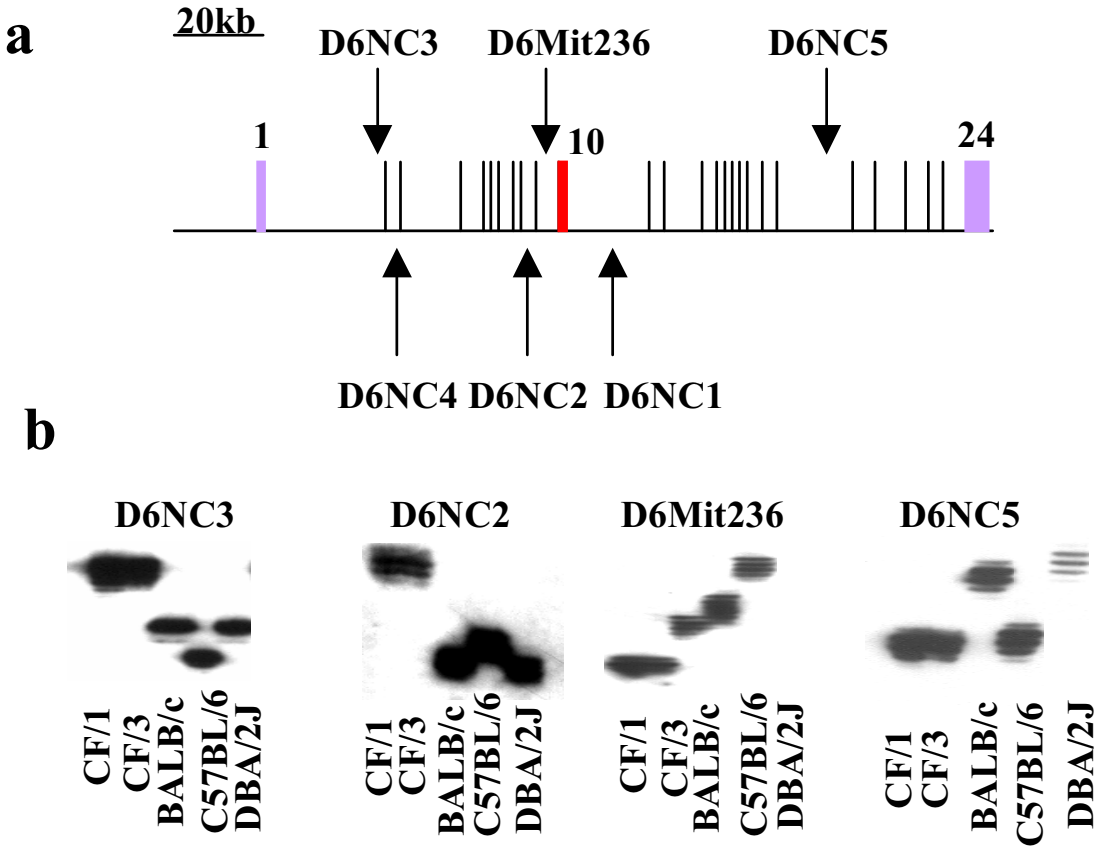


Figure 1
Generation of the *Cftr*$^{TgH(neoim)Hgu}$ mouse model. a) Insertional disruption of the murine *Cftr* gene and predicted gene structure as described by Dorin et al. (1992). Abbreviations: S, *Sal*I; H, *Hind*III; X, *Xba*I. Map position of the CFneo1 and CFneo2 products is also indicated. b) 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 *Xba*I+*Sal*I 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.



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
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 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.

Table 1: Expected Southern and microsatellite genotypes for animals backcrossed to the three inbred backgrounds.

	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**

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 *Sal*I 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- $Cftr^{TgH(neoim)Hgu}$ *Cftr* locus at least in mouse B.

Table 2: Genotypes of the animals with an excised vector. Mouse A in one chromosome, Mouse B in both chromosomes.

mouse	1.2H	D6NC3	D6Mit236	D6NC5
A	cf/n	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)
B	n/n	cf/cf (20/20)	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
Cftr-5012	CCT TCC ATG TAC CCC TCC TCA CTT CCC GGC ATA ATC CAA GAA AAT TG	5012 bp	Wild type
Cftr-5198	TGT GGG AAA TCC TGT GCT GAA A CTT CCG GCT CGT ATG TTG TGT T	5198 bp	mutant
Cftr-3736	CAC ACA ACA TAC GAG CCG GAA G TTT ATT GCC GAT CCC CTC AGA A	3736 bp	mutant
Cftr-3473	CTC GTG CTT TAC GGT ATC GCC TGC TGT AGT TGG CAA GCT TTG A	3473 bp	mutant

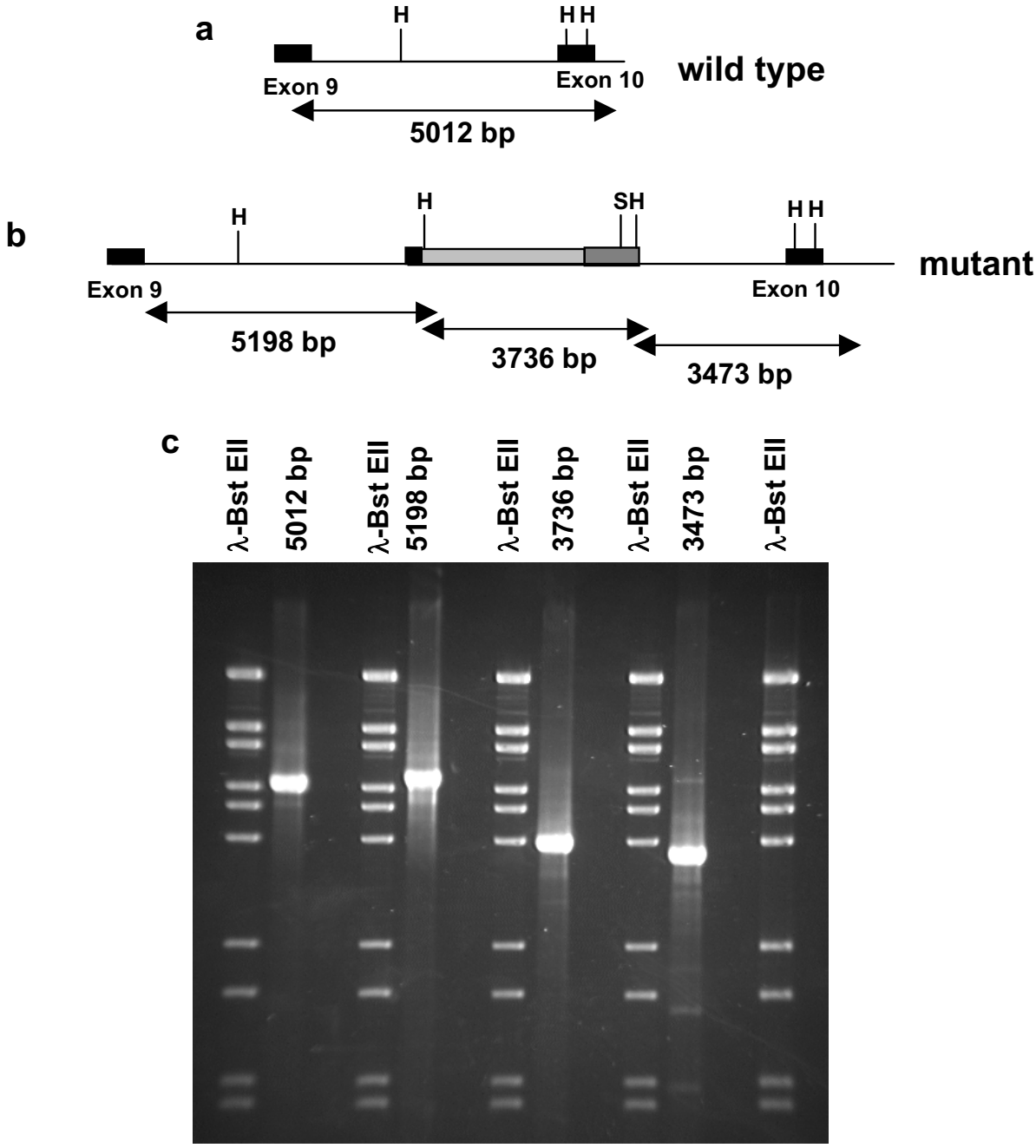


Figure 3
Long range PCR. a) Map position of the long range PCR product corresponding to the 5012 bp wild type sequence. **b)** Map position of the three long range products corresponding to the mutant allele. **c)** Representative agarose gel (1%) indicating the expected PCR products for all four primer 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 *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 *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 *Cftr* intron 9 where the vector was introduced via homologous recombination [12] the sequence was found to be 100% wild type *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 $Cftr^{TgH(neoim)Hgu}$ 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 *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 *Cftr* locus.

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)

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

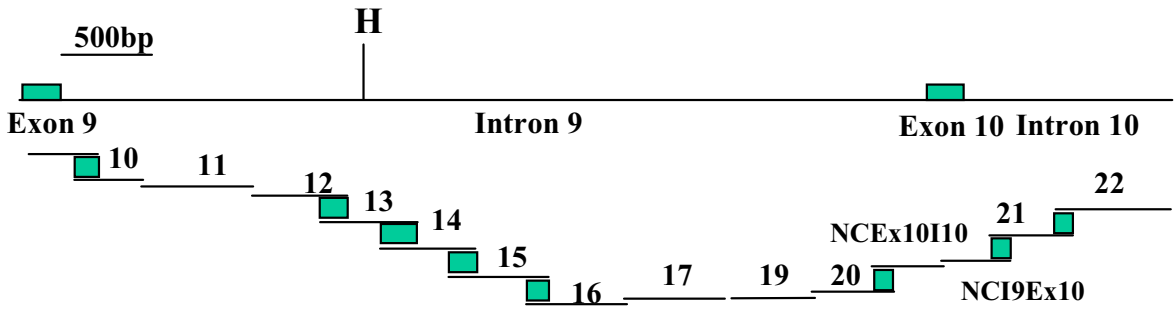


Figure 4
Primer walking. Straight lines represent the fragment amplified by each primer set, overlapping sequences are represented by boxes.

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 *Cftr*$^{TgH(neoim)Hgu}$ 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 [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- *Cftr*$^{TgH(neoim)Hgu}$ 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 [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 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.

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- *Cftr*$^{TgH(neoim)Hgu}$ 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 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-

Table 5: Mouse A and B littermate genotypes.

mouse	1.2H	D6NC3	D6Mit236	D6NC5
C	cf/n	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)
D	cf/n	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)
E	n/n	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)
F	cf/cf	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)
G	cf/cf	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)
H	cf/cf	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)
I	cf/cf	cf/cf (20/20)	cf/cf (20/20)	cf/cf (20/20)

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. $Cftr^{TgH(neoim)Hgu}$ 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 20–24°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$^{TgH(neoim)Hgu}$ 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.

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 [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 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 5 kb *Xba*I-*Sal*I 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 are listed in Table 6.

Table 6: Primer sequences used for the amplification of the intragenic *Cftr* microsatellites. The forward primer is 5'biotinylated.

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

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 $MgCl_2$, pH 9.5). The membrane was covered for 5 min with reaction buffer containing 10%(v/v) Sapphire II (Tropix) and 60 μl CDPstar (Tropix)in 50 ml assay buffer, followed by rinsing with a solution containing 1% v/v Sapphire II and 6 μl CDPstar in 50 ml assay buffer. Signals were exposed to Kodak XA-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-

TRE Technologies, WI USA). PCR products were amplified using primers described in Table 3 separated by 1% agarose gel electrophoresis and visualised under UV illumination, the optimal reaction mixture was thereafter chosen for further amplifications (Figure 3).

Neo PCR

Cftr intron 9-pIV3.5H vector and *neo -Cftr* intron 9 spanning primers, (Table 7) were amplified using PCR of 50 ng DNA template in a total volume of 30 μl 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.

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	
CFneo2-A	CCT GAT GTT GAT TTT GGG AGA	253 bp
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. PCR reactions were performed on DNA with inconsistent Southern and microsatellite insertional 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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