Loci of intestinal distress in cystic fibrosis knockout mice

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Haston, Christina K., and Lap-Chee Tsui. Loci of intestinal distress in cystic fibrosis knockout mice. Physiol Genomics 12: 79–84, 2003. First published November 19, 2002; 10.1152/physiolgenomics.00114.2002.—The strain-dependent survival of cystic fibrosis (CF) knockout mice has been used to map a modifier of CF, Cfml, in mice and, subsequently, in humans. To identify additional modifiers of the CF phenotype, in this study, the survival of F2 CF mice derived from a cross between congenic C57BL/6J CF and BALB/cJ CF heterozygotes was followed up to 12 wk of age. A genome-wide linkage scan completed in F2 CF mice revealed a chromosome 10 locus (P = 1.2 × 10−4) to predict for intestinal distress in CF male mice. An X chromosome locus for which non-Mendelian inheritance favoring B6 alleles in the surviving CF mice and BALB alleles in mice of a control population, was identified. The survival of female mice, both F2 CF and F2 control, was linked to loci on chromosomes 3 and 5. The identification of additional putative CF modifier loci may permit further genetic dissection of the complex CF phenotype.

modifier gene; transmission distortion

CYSTIC FIBROSIS (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR); a gene which encodes an epithelial cell chloride channel. In the classic manifestation of the disease the lungs, pancreas, sweat glands, and intestine are affected (4). The severity of the disease is highly variable due in part to the over 950 known mutations in CFTR and the existence of modifier loci (24). Apart from the pancreatic phenotype, the complications of CF cannot be predicted by CFTR genotype (24).

Mouse models have been made to investigate the complex CF phenotype (3) and have been used to uncover a CF modifier locus (20). As most CF mice succumb to intestinal distress within the first month of life, with a few able to live longer (1, 3), Rozmahel et al. (20) were able to use differences in allelic ratios at particular loci, between surviving CF mice and mice with the lethal intestinal defect, to map CF modifier gene one, Cfml. This result directed subsequent studies of the homologous region of the human genome, chromosome 19, and led to the identification of a genetic locus of susceptibility to meconium ileus in CF patients, CFM1 (25). CFM1 does not, however, predict for meconium ileus in all CF patients, indicating that other modifiers of this phenotype may exist.

In this study the survival of CF mice derived from a cross between C57BL/6J Cfr+/−/m1UNC and BALB/cJ Cfr+/-/m1UNC mice was studied. The existence of genetic factors influencing intestinal disease severity was inferred from an unequal representation of parental alleles among the survivors. To ensure that a severe phenotype was scored, the mice were fed a liquid diet and survival was recorded up to the age of 12 wk. Genotyping of F2 CF and control mice was completed to identify regions of the genome associated with CF mouse survival and those associated with transmission distortion in a general B6 and BALB cross.

MATERIAL AND METHODS

Mice. Congenic C57BL/6J (B6) CF and BALB/cJ (BALB) CF heterozygote mice were generated by repeated backcrossing from the original Cfr knockout (m1UNC) mutant mice provided by Dr. Bev Koller (23). At the time of these experiments, the B6 and BALB Cfr heterozygote breeders were at the 23rd and the 20th backcross generation, respectively. The breeders were established to be B6 and BALB, respectively, at all 66 genome-wide evaluated microsatellite markers. The congenic heterozygotes B6 and BALB CF (+/−) mice were crossed to create F1 mice. F1 Cfr+/− mice were intercrossed to create F2 CF (−/−) mice. In this report, F1 and F2 mice are named with the parental dam strain name followed by that of the sire.

The Cfr genotype of the mice was determined using a previously described PCR assay (9) and genomic DNA isolated from the tails of mice, which were clipped at 18 days of age. All CF mice were maintained on liquid diet (Peptamen, Ref. 10) from the age of 19 days until death. The control mice were fed either liquid diet or solid chow (Pro Lab 1000 rodent chow). All mice were housed in micro-isolator cages in a specific pathogen-free room and handled according to the standard husbandry of the animal facility at the Hospital for Sick Children. The studied mice were killed, by cervical dislocation, when in distress (intestinal obstruction) or at the age of 12 wk (survivors).

Genotyping. DNA was prepared from the tail clips and spleen tissue harvested from F2 animals at death. Mouse genetic markers, identified by Dietrich et al. (6), were used as primers to produce polymorphic PCR products at 186 specific loci, representing chromosomes 1–19 and X. The PCR products were separated by 3% agarose gel electrophoresis and visualized with ethidium bromide staining according to standard protocols.

Inheritance of B6 or BALB alleles. A survey of the whole mouse genome for non-Mendelian transmission of B6 and
BALB alleles among the F2 CF animals of the above-described cross was performed with a panel of 156 markers at an average spacing of 10 cM based on Mouse Genome Database data (14). All 214 F2 CF surviving mice were typed, and the data revealed nine regions of the genome with unequal allelic inheritance, by $x^2$, with $P < 0.05$. This level of significance ($P < 0.05$) was not used to identify linkage, but to isolate potential linkage regions suitable for further investigation in the CF and control mice. The F2 CF mice were genotyped with a total of 30 additional markers in these regions. The Cftrm1 region on chromosome 7 (10th region) was also typed to assess the inheritance of B6 and BALB alleles at this locus. In addition, 58 of 62 F2 CF mice that were found dead in the study (between the ages of 3 and 12 wk) were genotyped for a total of 70 markers in the 10 regions (the DNA had degraded for 4 dead mice), along with two F2 CF mice that were killed early due to distress. Ninety-six F2 control mice were genotyped for a total of 70 markers in the 10 regions. The controls (45 females, 51 males) were taken from 33 different litters, and the $Cftr$ genotypes of (+/+ , $n = 34$) and (+/−, $n = 62$) were represented.

Differences in genotype ratios at specific loci between the mouse groups (CF vs. control, and CF survivors vs. nonsurvivors), and compared with Mendelian expectations, were assessed by $x^2$ analysis using Microsoft Excel software. Linkage was assessed using the standards proposed by Lander and Kruglyak (11) and the probabilities calculated from the $x^2$ test. Interaction between putative loci was also evaluated through $x^2$ testing, as used by others (17).

RESULTS

Inheritance of Cftr knockout allele. To study the survival of congenic B6 and BALB CF mice and to facilitate a genetic evaluation of factors contributing to this phenotype, CF heterozygote mice were intercrossed to produce B6, BALB, and (B6 × BALB)F2 CF mice. At weaning the mice of these litters ($n = 2,620$) were genotyped for $Cftr$ and the 316 CF mice identified was significantly fewer than the Mendelian expectation of one-fourth of the total, or 655 mice. The ratio of mice with genotype +/+ to mice with genotype +/− was consistent with the expected 1:2 ratio ($P = 0.67$). This same ratio of +/− to +/+ genotypes was observed in all crosses and for both sexes (data not shown).

The under-representation of the $Cftr$ −/− genotype was most pronounced in the generation of BALB and B6 CF mice. In the litters of the parental strains, 18 B6 CF mice were identified by genotyping (of 82 expected, 22%), as were 20 BALB CF mice (of 153 expected, 13%). The number of non-CF mice, by their genotypes at weaning, was assumed to represent three-fourths of the litter, and the expected CF mouse total was calculated from this data. The B6 litters were 47.8% male, and the BALB litters were 51.3% male. Intercrossing the strains to produce F2 mice increased the level of CF mouse survival to weaning, relative to B6 or BALB congenic survival, as 278 F2 CF mice (of 518 expected or 54%) were identified by genotyping.

Survival of CF mice. The survival of the B6, BALB, and F2 CF mice, identified by their genotype at weaning, was followed to the age of 12 wk. The parental CF strains did not differ ($P = 0.20$) in their postweaning survival, as 70% of the BALB mice (14 of 20) lived to the death time of 12 wk, as did 78% (14 of 18) of the B6 mice. A difference in survival by the sex of the animals was, however, apparent, as five of the eight BALB CF male mice died before the age of 12 wk, whereas only one of twelve BALB CF females died in this time (P = 5.7 × 10⁻⁵). The postweaning survival of the B6 CF mice did not depend on sex, as the same number B6 CF males and females died between weaning and the end of the experiment.

A parental influence on the survival of F2 mice was identified, with offspring of a (BALB × B6)F1 father less likely to survive to 12 wk than offspring of a (B6 × BALB)F1 father ($P = 0.02$, data not shown). The overall survival of the F2 CF mice was similar to that of the parental CF mice; 77% (214 of 278) of F2 CF mice lived to the death time of 12 wk. The phenotype in F2 mice did not strongly depend on gender ($P = 0.09$, data not shown). Two F2 CF mice were killed at 8 wk of age because of severe distress determined, at autopsy, to be due to an intestinal block. No control mice died during the study.

Loci of non-Mendelian inheritance in CF surviving mice. A genome scan was completed in CF mice surviving to the age of 12 wk, as an initial step to identify genes that contribute to the survival of CF mice. The genotypes of these mice, at each locus, were compared with Mendelian expectations, and genomic regions that differed from the expectation ($P < 0.05$) were further investigated. We chose the $P < 0.05$ level for further investigation to be inclusive of all potential linkage regions, but our interpretation of the final data set is based on the standards for linkage in this type of cross (11).

The genome scan, completed with 156 markers, uncovered nine regions of non-Mendelian inheritance of parental alleles in the F2 CF survivors at the level of $P < 0.05$. The mice were genotyped with an additional 30 markers in these regions, and the markers presenting the maximal transmission distortion at each region are shown in Table 1. As shown in Table 1, the surviving female CF mice had an excess of BALB alleles at loci on chromosomes 3, 4, and 5 and an excess of B6 alleles at loci on chromosomes 1, 17, and 19. The remaining three regions of transmission distortion were identified in male mice, with an excess of BALB alleles at a locus on chromosome 4 and an excess of B6 alleles at loci on chromosomes 10 and X. The X chromosome results were observed in F2 mice made by all four possible F1 intercrosses (data not shown).

The potential modifier regions were further investigated by determining the genotypic ratios in F2 CF nonsurvivors and non-CF control mice (+/+ and +/−), at the regions. The genotypic ratios of the non-CF control mice were used to ascertain whether the putative distortion measured in the CF mice (Table 1) was specific to CF mice.

Putative CF modifier locus: postweaning survival. From the data in the parental mice, fewer BALB CF males lived from weaning to the age of 12 wk than B6 males. To map this difference in survival, we compared the genotypic ratios of the F2 CF surviving mice to
those of nonsurvivors at each of the loci listed in Table 1. The data of males and females were considered separately because of the sex difference in survival of BALB CF mice. In the same sex male the genotypic ratio (in the form B6/B6:B6/BALB:BALB) for the CF survivors, 27:56:11, was significantly different (P < 0.0016, the standard for suggestive linkage, Ref. 11) at marker D10Mit194 (P = 1.2 × 10⁻⁴). This locus spans ~25 cM on proximal chromosome 10. This difference in genotypic ratio at D10Mit194 was not seen in the female mice, as shown in Table 1. The genotype ratios of the surviving and nonsurviving CF mice did not significantly differ (P > 0.0016, the standard for suggestive linkage, Ref. 11) at any of the remaining regions (data not shown); thus we were able to map one locus influencing (B6 × BALB)F2 CF mouse survival from 3 to 12 wk of age.

**Putative CF modifier locus: survival to weaning.** The survival of (B6 × BALB)F2 CF mice to weaning exceeded that of B6 or BALB CF mice, which indicates that genetic factors influence this trait. To map the survival to weaning phenotype, the genotypes of the F2 CF survivors and nonsurvivors, at each of the loci in Table 1, were combined to create a set of genotypes of CF mice which had survived to weaning (278 F2 CF mice). The allelic ratios of this CF set were then assessed for Mendelian transmission of genotypes, and where the ratios were found to differ from expectations, modifier loci which enabled the F2 CF mouse to live to weaning were hypothesized to exist. Non-Mendelian inheritance of genotypes in CF mice was identified for loci on chromosomes 1, 3, and X, as shown in Table 2. These loci are potential CF modifiers if shown to not predict for the survival to weaning of a control mouse.

To determine whether the non-Mendelian inheritance ratios identified in the F2 CF mice were specific to CF mice, we genotyped 96 control mice for the regions listed in Table 1. The allelic frequencies identified in the control mice, which are relevant to the investigation of potential CF modifier loci, are listed in Table 2. As shown in Table 2, three regions of non-Mendelian inheritance were identified in control mice. For each of these regions, on chromosomes 3, 5, and X, the non-Mendelian inheritance of genotypes was identified in both Cfrt+/+ and +/- mice (data not shown). As with the data of CF mice on the X chromosome, these results were independent of the direction of F1 parental cross used to produce the F2 mice (data not shown).

### Table 1. Allele frequency in (B6 × BALB)F2 CF survivor mice

| Peak Marker | Males | | | | | Females | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | B6 Alleles* | BALB Alleles | P value | B6 Alleles | BALB Alleles | P value |
| D1Mit504 | 107 | 87 | 0.15 | 142 | 94 | 0.0018 |
| D3Mit189 | 94 | 110 | 0.062 | 91 | 137 | 2.3 × 10⁻³ |
| D4Mit145 | 101 | 95 | 0.67 | 99 | 135 | 0.019 |
| D4Mit254 | 68 | 98 | 0.02 | 106 | 102 | 0.78 |
| D5Mit239 | 97 | 99 | 0.89 | 96 | 138 | 6.0 × 10⁻³ |
| D10Mit194 | 113 | 83 | 0.032 | 108 | 124 | 0.29 |
| D17Mit57 | 105 | 81 | 0.078 | 130 | 100 | 0.048 |
| D19Mit71 | 85 | 103 | 0.19 | 132 | 98 | 0.025 |
| DXMit16 | 59 | 33 | 1.3 × 10⁻⁴ | 112 | 108 | 0.79 |

*Number in F2 mice with this genotype (the total number is not constant due to missing genotypes). †For male mice the X chromosome genotypes are B6 or BALB. ‡Compared to the Mendelian expectation of a 1:2:1 ratio of genotypes. §D7Mit340 has been mapped at 1.2 cM and Cfm1 at 1.5 cM on mouse chromosome 7 (Mouse Genome Database, 2002).

### Table 2. Genotype frequency in (B6 × BALB)F2 mice

<table>
<thead>
<tr>
<th>Peak Marker</th>
<th>Group</th>
<th>n</th>
<th>B6/B6*</th>
<th>B6/BALB</th>
<th>BALB/BALB</th>
<th>P value‡</th>
<th>P value, CF vs. controls</th>
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</thead>
<tbody>
<tr>
<td>D1Mit504</td>
<td>CF</td>
<td>278</td>
<td>82</td>
<td>145</td>
<td>45</td>
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<td>45</td>
<td>23</td>
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<td>D3Mit189</td>
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<td>140</td>
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<td>0.44</td>
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<tr>
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<td></td>
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<td>73</td>
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<td>0.29</td>
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<td>0</td>
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<td>3.1 × 10⁻¹⁰</td>
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<td>34</td>
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<tr>
<td>CF females</td>
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<td>79</td>
<td>25</td>
<td>0.13</td>
<td>1.4 × 10⁻⁶</td>
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<td>23</td>
<td>19</td>
<td>0.0033</td>
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<tr>
<td>D5Mit239</td>
<td>CF</td>
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<td>64</td>
<td>130</td>
<td>79</td>
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<td>19</td>
<td>44</td>
<td>33</td>
<td>0.093</td>
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<tr>
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<td>64</td>
<td>47</td>
<td>0.071</td>
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<td></td>
</tr>
<tr>
<td>Control females</td>
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<td>5</td>
<td>19</td>
<td>21</td>
<td>2.0 × 10⁻³</td>
<td></td>
<td></td>
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<tr>
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<td>126</td>
<td>65</td>
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<td>50</td>
<td>17</td>
<td>0.21</td>
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</table>
shown). At the loci of Table 1 not listed in Table 2, Mendelian inheritance ratios were identified for the genotypes of the control mice (data not shown).

The putative CF modifier regions identified based on transmission distortion in CF mice, listed in Table 2, were further evaluated by comparing the ratios of genotypes of CF mice to those of control mice (Table 2, last column). The genotypic data sets of CF mice were found to not differ from those of controls for $D1Mit504$ ($P = 0.21$) and $D3Mit189$ ($P = 0.44$); thus the transmission distortion originally identified in CF mice was found to be independent of $Cfrt$ genotype. At the putative X chromosome modifier locus the ratio of alleles of male F2 CF mice (excess B6 alleles) was found to differ from that of controls (excess BALB alleles), with the maximal difference at $DXMit16$ ($P = 3.1 \times 10^{-10}$), see Table 2. The interval for this distortion, defined by a decrease of the LOD score by a value of one on either side of $DXMit16$ (Ref. 12), extends from $DXMit1$ (at 29 cM) to $DXMit64$ (at 45 cM). This difference in inheritance of parental genotypes was also detected in the female mice ($DXMit166$, $P = 1.4 \times 10^{-6}$, 10 cM proximal to the peak identified in males), as the genotypic ratio of CF mice differed from that of controls. In the female mice, however, the non-Mendelian inheritance of genotypes was evident in the controls only, as shown in Table 2. Thus an X chromosome locus potentially modifies the survival of an F2 CF mouse to weaning.

No statistical interaction was evident between the two potential CF modifier loci, as the genotypes at marker $D10Mit194$ were inherited independently of those at $DXMit16$ ($P = 0.82$) in male mice.

Finally, regions of transmission distortion that resulted in an excess of BALB alleles at specific loci on chromosomes 3 and 5 were identified in both CF and control female mice (see Table 2). The ratios of alleles of the combined CF and control dataset were shown to be significantly different from 1:1 in this B6/BALB cross for these regions of chromosomes 3 (peak marker $D3Mit189$, $P = 1.7 \times 10^{-4}$, region length 7 cM) and 5 ($D5Mit239$, $P = 8 \times 10^{-4}$, 19 cM). In addition, only one female mouse (of 163) was homozygous B6 at both $D3Mit189$ and $D5Mit239$. For the female mice on chromosome 5, the transmission distortion observed was influenced by the F1 parental mice used in the crosses. For the surviving (CF) female mice, non-Mendelian inheritance ratios were observed in offspring of the (BALB × B6)$F1$ × (B6 × BALB)$F1$ only, whereas in the control female mice, non-Mendelian inheritance ratios were observed in offspring of the (BALB × B6)$F1$ × (BALB × B6)$F1$ only (data not shown).

**Cystic fibrosis modifier 1 (Cfm1).** The chromosome 7 genotypic ratios in the CF and control mice were investigated as a potential CF modifier region, based on the mapping of $Cfm1$ (20). The genotypic ratios of the CF mice, in this study, were consistent with Mendelian expectations ($P = 0.42$, see Table 2), at a proximal chromosome 7 marker mapped near $Cfm1$; thus this genotype does not predict for CF mouse survival in this cross. In 45 F2 female control mice, however, there was an excess of homozygous B6 genotypes at $Cfm1$ ($P = 0.058$), which was enhanced at a marker ($D7Mit55$, $P = 0.006$) ~14 cM distal to $Cfm1$. When this discrepancy was evaluated by $Cfrt$ genotype (mapped to mouse chromosome 6), of the female mice that were $Cfrt$ and seven were homozygous B6 at $D7Mit55$, eleven were heterozygous and none were homozygous BALB. Thus this preliminary evidence indicates the ratio of genotypes of female control mice, near $Cfm1$, is dependent on $Cfrt$ genotype ($P = 0.02$). This difference was not seen in the male control mice (data not shown).

**DISCUSSION**

A goal of modifier gene mapping studies, such as this, is to identify the loci of genes through which the disease causative gene (here, $CFTR$) acts to produce the phenotype. Here we presented evidence for modifier genes which affect CF intestinal disease, mapped to chromosome 10, and potentially, the X chromosome. The putative CF modifiers identified in this study require further investigation for confirmation, ultimately including human studies.

Our evaluation of the survival of congenic B6 and BALB CF mice showed BALB CF male mice to have higher postweaning lethality than both the BALB CF female mice and B6 CF mice. The mapping study revealed a chromosome 10 locus in which inheritance of the BALB/BALB genotype was associated with a higher rate of lethality in F2 CF male mice. The few mice killed due to impending death in the postweaning period presented intestinal blocks, indicating that late survival in CF mice on liquid diet is limited by intestinal complications, as reported by Kent et al. (10). Gomyorey et al. (8) and Rozmahel et al. (20) have investigated the intestinal phenotype of long-surviving CF mice and identified improved chloride secretion in these mice compared with those succumbing to distress. No candidate genes with potential roles in CF intestinal physiology, such as chloride channels or mucins (18), have, however, been mapped to the region on chromosome 10 (7, 14). Mouse chromosome 10 is also not known to contain imprinted genes (14), so the parental effect on F2 CF mouse survival, which suggests that imprinting may affect this phenotype, may be separate from this locus.

The potential CF modifier region on chromosome 10 is distinct from $Cfm1$, which was mapped to chromosome 7 in CD1/129 mice (20). The identification of additional loci influencing the intestinal phenotype of CF mice, derived from models of differing inbred strains, illustrates the utility of investigating knockout mice with different genetic backgrounds when dissecting factors influencing complex traits. The modifier evidence presented in this mouse model is, however, consistent with that of the previous study, as the supporting evidence provided in the mapping of $Cfm1$ (20) showed that both B6 and BALB alleles at $Cfm1$ spare the intestinal phenotype in CF mice, relative to 129/Sv alleles.

In this study a higher survival rate to weaning was identified in (B6 × BALB)$F2$ CF mice, relative to B6 or
BALB CF mice, which is similar to previous studies of outbred (genetically mixed) Cftm1UNC/m1UNC mice (9, 10). The CF mice not surviving to weaning are presumed to have succumbed in both the prenatal and postnatal periods. Our birth records indicated that a fraction of the litters died before weaning (postnatal), but accounting for these does not increase the number of CF mice to the expected 25% of the litters; thus prenatal lethality is presumed to have occurred. Thus the phenotype of survival to weaning is from death due to intestinal distress in the perinatal period as in Refs. 20 and 23, or death in utero, which may not be due to intestinal distress specifically.

We identified a region of the X chromosome with non-Mendelian inheritance of alleles in CF mice which may influence the survival of F2 CF mice to weaning. This locus appears to affect CF and control mice differently, with B6 alleles sparing the phenotype in CF mice and BALB alleles over-represented in the control mice. The potential of the inheritance of B6 alleles to contribute to F2 mouse survival is consistent with the higher survival rate of B6 CF mice to weaning (22%) than BALB CF mice (13%).

Survival due to inheritance of X-linked transmission distortion loci has been identified in several (non-CF) mouse crosses (5; reviewed in Ref. 13) and in human studies (16). The causative genes producing non-Mendelian inheritance have not been uncovered, but a mechanism of embryonic lethality due to specific allelic combinations has been proposed. In this study, the transmission distortion identified for X chromosome genotypes is consistent with the mechanism of embryonic lethality, and not meiotic drive, as it was independent of the direction of the parental cross used to produce the F2 mice (2, 17). The present findings are related to the prior reports, further to the X chromosome involvement, in that the B6 allele is disfavored in control mice (here relative to BALB, others relative to DDK strain genotype) and that the distortion may depend on the sex of the mice. In this study, although the transmission distortion pattern was similar in male and female mice, the loci controlling the effect may be distinct, as their positions in male and female mice do not completely overlap and transmission distortion was not evident in CF female mice. Distinct from the prior work, however, is the fact that CF mice were also studied and in the CF mice the inheritance of B6 alleles spares the preweaning lethality. It is possible, due to the nature of studies of congenic mice, that the X-linked transmission distortion in the CF mice is due to homozygosity of particular 129/Sv alleles in the donor region of chromosome 6, and not the CF mutation. Further study is required.

Last, the unequal inheritance of B6 and BALB alleles in the mice of this study also led to the identification of putative loci of transmission distortion, on chromosomes 3 and 5, in female mice. These loci do not overlap with those detected in prior distortion studies completed in mice (5, 13, 17, 21, 22) or humans (15, 19), but the present study is the first report of this type in a B6/BALB intercross. Croteau et al. (2) investigated transmission distortion in a B6/BALB backcross but did not evaluate the linkage regions presented here. The regions of transmission distortion uncovered in that study (on proximal chromosome 6, distal chromosome 7, and distal chromosome 12) were not distorted in the CF mice and thus were not evaluated in the control mice here. As B6 mice are both viable and homozygous B6 for all loci, the under-representation of this genotype on chromosomes 3 and 5 in female (B6 × BALB)F2 mice is most likely indicative of an effect in mixed background mice only.

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