Bleomycin hydrolase and a genetic locus within the MHC affect risk for pulmonary fibrosis in mice

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Susceptibility to pulmonary fibrosis following environmental insults or cytotoxic cancer therapies has a genetic component. In mouse strains differing in susceptibility to bleomycin-induced lung fibrosis, we show highly significant linkage to only two loci. The first locus on chromosome 17 in the major histocompatibility complex (MHC), LOD = 17.4, named Blmpf1, is highly significant in both males and females, and accounts for approximately 20% of the phenotypic variance. We confirmed the presence of Blmpf1 in MHC congenic mice and narrowed the region to 2.7 cM in a reduced MHC congenic strain. The second locus on chromosome 11, LOD = 5.6, named Blmpf2, is significant in males only. A model including an interaction between Blmpf1 and Blmpf2 best fit the data in males. We confirmed Blmpf2 in a chromosome substitution strain, C57BL/6J-11C3H, and found that its presence reduces the severity of fibrosis. Functional studies of bleomycin hydrolase activity indicate that this enzyme modulates bleomycin-induced pulmonary fibrosis, suggesting that it may be a candidate gene for Blmpf2. The data suggest sex-specific models of susceptibility to bleomycin-induced lung fibrosis, with an interaction between Blmpf2 and Blmpf1 for the more susceptible males and Blmpf1 as the major locus in females. A putative mechanism for the interaction between the two loci in males is that bleomycin hydrolase functions as an MHC class I epitope-processing protease.

INTRODUCTION

Pulmonary fibrosis is the endstage of a pathologic process initiated by disparate environmental insults, including ozone and particle exposure (1,2). Pulmonary fibrosis following lung irradiation or chemotherapy for the treatment of cancer results in significant morbidity and mortality in a subset of cancer patients (3,4). Currently, few effective treatments exist for pulmonary fibrosis after any insult (5). It remains a debilitating and potentially life-threatening condition. The need to avert pulmonary fibrosis limits the dose of radiation and some chemotherapeutic agents that can be given and thus potentially decreases the effectiveness of treatment (6,7). Identifying the genes affecting susceptibility could allow dose escalation and provide a higher probability of cure (8).

Both clinical and experimental studies indicate that pulmonary fibrosis has a genetic component (9,10). Inbred strains of mice differ in their propensity to develop lung fibrosis after exposure to bleomycin (BLM), which is a model that can be used to identify genetic factors influencing susceptibility to pulmonary fibrosis (11–13). Previously, in a study of a cross between C3Hf/Kam (C3) mice, which are resistant to BLM, and C57BL/6J (B6) mice, which are susceptible, we estimated the heritability of BLM-induced fibrosis to be 53% in male and 54% in female mice (12). In MHC congenic mice of different inbred strain backgrounds, strain-specific genetic factors and the major histocompatibility complex (MHC) genotype influence susceptibility to pulmonary fibrosis (11). The region encompassing the MHC has been identified in other lung response linkage studies, including susceptibility to asthma (14) and to radiation-induced (15), ozone-induced (16) and particle-induced lung inflammation and fibrosis (2), suggesting a common genetic basis for pulmonary fibrosis independent of the causative agent. The linkage region reported in all of these studies suggests that a genetic component within the MHC affects susceptibility to pulmonary fibrosis.

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studies is too large, however, to allow positional cloning of causative gene(s). Thus the precise genetic basis underlying these linkage studies has yet to be defined. Elucidation of the fibrotic pathway may also be applicable to understanding the pathogenesis of lung cancer, as the processes are known to be linked (17) and susceptibility loci overlap (18).

In this paper, we report the results of a genome-wide screen for factors influencing susceptibility to BLM-induced pulmonary fibrosis in B6C3F2 mice derived from susceptible, C57BL/6J(B6) and resistant C3Hf/Kam (C3) strains. We completed further studies in congenic strains and a chromosome substitution strain to confirm the two loci identified. Finally, the enzymatic activity of the candidate gene bleomycin hydrolase (BH) was investigated.

RESULTS

Mapping of susceptibility loci

To identify regions of the genome linked to BLM-induced lung fibrosis, we genotyped the 94 phenotypically extreme mice of 361 (19). These mice included those with zero fibrosis (n = 45, 20 females and 25 males) and those with fibrosis greater than 3% of the lung (n = 49, 27 females and 22 males). The initial scan using MapManager was completed with 190 markers spanning the genome with an average intermarker distance of ~10 cM. This scan revealed two regions of the genome with LOD scores exceeding 2.8, one on chromosome 17 and the second on chromosome 11. Additional mapping was then completed in these regions by genotyping all the F2 mice with 13 markers on chromosome 17 and 21 markers on chromosome 11.

Significant linkage was established for these loci (Fig. 1). The quantitative trait locus (QTL) identified on chromosome 17 between markers D17Mit198 and D17Mit16 showed highly significant evidence for linkage in both male (LOD = 10.0) and female mice (LOD = 8.2). The total LOD score of 17.4 was not observed in permutation analysis and so has a P-value of less than 1/5000 genome scans. We named it BLM-induced pulmonary fibrosis 1, or Blmpf1. This locus accounted for ~20% of phenotypic variance of the F2 generation. MapManager analysis indicates that the inheritance is nearly additive (Table 1). At the peak marker of Blmpf1, the mean fibrosis scores of the mice were 4.53% for the B6/B6 genotype, 1.42% in heterozygous mice and 0.065% in the homozygous C3H/C3H mice. This locus, Blmpf1, accounts for 38% (20%/53%) of the genetic contribution to this trait. The second locus, Blmpf2, yielded a combined LOD score for males and females of 3.3 at marker D11Mit272. Permutation analysis using markers D11Mit272 and D11Mit310 assigned a significance level of 6 per 10 000 to this result. Linkage analyses by sex showed detectable evidence on males only, LOD = 4.1 at D11Mit 272. This QTL contributes 8.9% of phenotypic variance in F2 mice and was inherited additively (Table 1). We found weaker evidence for a third locus on chromosome 8 at markers D8Mit242 and D8Mit271, with a peak LOD score of 2.78.

Using MapManager, we re-analyzed the phenotypic and genotypic data after ‘conditioning’ on Blmpf1 and Blmpf2. For female mice conditioned on Blmpf1, the LOD score for Blmpf2 remained unchanged and no other loci with LOD scores exceeding 2.8 were detected. For the male mice, the LOD score for Blmpf2 increased to 5.6 after accounting for the significant linkage of Blmpf1. After conditioning on Blmpf1, the maximum LOD score on chromosome 8 decreased to 2.37, 5 cM telomeric to D8Mit242. No other loci with LOD scores exceeding 2.8 were detected in either the male or female mice after conditioning on Blmpf1. We used analysis of variance (20) to test for interactions between the identified QTL. This analysis revealed the presence of a statistically significant interaction (F4,121 = 76.8; P < 0.0001) between Blmpf1 and Blmpf2 (Tables 2 and 3). When compared with the doubly heterozygous mice, a highly significant interaction
(P = 0.0006) was noted for the animals homozygous for the C57BL/6J marker alleles at D11Mit272 and D17Mit16, and these animals had 5.03% (+1.44%) more fibrosis than predicted in the absence of any interaction. There was also a slight decrease of −2.90% (+1.36%) from predicted in the fibrosis among animals that were homozygous for the C3H/Kam alleles at D11Mit272 and homozygous for C57BL/6J markers at D17Mit16 (P = 0.03).

Owing to the a priori suggestion of X-linkage from the inheritance study (12), 11 X chromosome markers were included in the genome scan. No linkage was identified for markers on the X chromosome, in either sex, despite mapping at a conservative 7 cM interval. Also, no linkages were identified for regions on chromosomes 1, 6 and 18 (data not shown), for which we show significant QTLs in linkage studies using a radiation-induced pulmonary fibrosis model. Blmpf1, however, mapped to the same region in the MHC as a locus for radiation-induced lung fibrosis (21).

**Blmpf1 is located within the MHC**

As the MHC haplotype of C3 mice is H2k and that of B6 mice is H2b, we confirmed that Blmpf1 is in the MHC by testing fibrosis susceptibility in MHC congenic mice. The donor regions of both congenic strains, C3.SW-H2b/SnJ and B6.AKR-H2k, overlap the distal 1 cM of Blmpf1, between D17Mit16 and D17Mit13 (Fig. 2). The positioning of Blmpf1 within the donor region of C3.SW-H2b/SnJ mice was confirmed in both sexes, as both male and female mice of this strain developed fibrosis after BLM treatment, while the C3Hf/Kam mice developed fibrosis only in the female sex (Fig. 3, Table 4). The fibrosis score in the C3.SW-H2b strain (%PF is 9.2 in males and 6.9% in females) was not significantly different from that in the fibrosis-prone C57BL/6J (H2b) strain (%PF is 6.8 for males and 4.04 for females, P = 0.46 and 0.98 respectively). This finding was further supported by data from the second MHC congenic strain, B6.AKR-H2k, as neither these mice nor the C3Hf/Kam mice developed fibrosis after BLM treatment and thus are distinct from the fibrosis-prone C57BL/6J mice.

**Blmpf1 narrowed to 2.7 cM region in a reduced MHC congenic strain**

The congenic data support the conclusion that the introgression of H2b MHC onto a C3 background results in fibrosis susceptibility. To narrow the linkage region, we generated two congenic lines containing subregions of the introgressed MHC. One strain did not develop fibrosis following BLM treatment and one strain did, enabling us to reduce the region of Blmpf1 to a 2.7 cM (Fig. 3, Table 4) region of the MHC between markers D17Mit175 at 17.7 cM and D17Mit148 at 20.4 cM (Fig. 2). We designated this strain C3.SW-H2b/C3H-D17Mit175−D17Mit147 (C3H-H2b reduced congenic). Fibrosis in the C3H-H2b reduced congenic mice was not significantly different from that in either the fibrosis-prone B6 strain (P = 0.39 and 0.78 for males and females respectively) or the C3.SW-H2b congenic strain (P = 0.21 and 0.66 for males and females respectively), confirming the presence of Blmpf1 (Table 4). Furthermore, fibrosis was similar in the male and female C3H-H2b reduced congenic mice (Table 4).

**Blmpf2 reduces bleomycin-induced pulmonary fibrosis**

We confirmed the existence and sex specificity of Blmpf2 in a chromosome substitution strain (consomic strain) (22), constructed using a speed congenic approach (23) to replace chromosome 11 in the B6 strain with chromosome 11 from the C3 strain. This strain was designated C57BL/6J-11c3H(B6-11c3H). Male B6-11c3H mice developed significantly less fibrosis than B6 male mice, %PF 0.9 and 10.7, respectively, P = 0.00007 (Table 4, Fig. 4). These data also show an interaction between Blmpf1 and Blmpf2, as the B6-11c3H mice are homozygous B6 at the chromosome 17 QTL and yet developed minimal fibrosis (Table 4). The phenotype in the male B6-11c3H mice was similar to that in male F2 mice with the same genotype (%PF = 0.9 and 1.6, respectively, P = 0.2) (Table 4), which indicates the effects of Blmpf2. Furthermore, gene dosage had a clear effect; the mean fibrosis scores by genotype were 4.3% for the B6/B6 phenotype, 1.5% for the heterozygous mice and 0.9% for the C3/C3 genotype, so the effect of the C3 allele is to reduce the severity of the phenotype.

For female B6-11c3H mice, the fibrosis score was significantly less than in the female B6 mice, %PF = 2.5 and 6.6 respectively (P = 0.00005), and was not significantly different from the %PF (3.98% P = 0.3) in the genotypically identical F2 mice, although the number of these mice was small (Fig. 4, Table 4).

### Table 1. Models of the inheritance of identified loci

<table>
<thead>
<tr>
<th>Marker name</th>
<th>Subset</th>
<th>n</th>
<th>LOD</th>
<th>Additive LOD</th>
<th>P-value</th>
<th>Dominant LOD</th>
<th>P-value</th>
<th>Recessive LOD</th>
<th>P-value</th>
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<td>D17Mit16</td>
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<td>356</td>
<td>17.4</td>
<td>16.0</td>
<td>0.01</td>
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<td>4.3</td>
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<td>8.6</td>
<td>0.01</td>
<td>3.9</td>
<td>1.2 × 10⁻⁷</td>
</tr>
<tr>
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<td>Mice</td>
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<td>5.6</td>
<td>5.3</td>
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<td>0.02</td>
<td>2.3</td>
<td>9.8 × 10⁻⁵</td>
</tr>
<tr>
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<td>8.2</td>
<td>7.7</td>
<td>0.13</td>
<td>7.0</td>
<td>0.02</td>
<td>2.9</td>
<td>7.9 × 10⁻⁷</td>
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<tr>
<td>D11Mit272</td>
<td>Mice</td>
<td>94</td>
<td>0.13</td>
<td>0.04</td>
<td>0.53</td>
<td>0.11</td>
<td>0.75</td>
<td>0.0</td>
<td>0.44</td>
</tr>
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</table>

*LOD score calculated assuming no genetic model (free).  
P-values were obtained by converting the difference in LOD scores between the general model and one of the restricted inheritance models (e.g. additive), multiplying by 4.6 to convert to a chi-squared deviate and then comparing with the chi-squared distribution.  
LOD score calculated conditioned on D17Mit16.
Bleomycin hydrolase activity correlates with the phenotype

To determine whether the enzyme that detoxifies BLM, BH, is a candidate gene for Blmpf2, we assessed the activity of this enzyme in both males and females of the C3, B6 and B6-11C3 strains at times between 7 and 28 days after treatment with BLM in three independent experiments. BH activity progressively and significantly decreased, reaching a nadir at 21 days after BLM treatment, in both male and female fibrosis-prone B6 mice compared with their untreated counterparts ($P = 0.00048$ and $0.00046$) (Fig. 5), in agreement with previously reported data (24). Likewise, BH activity peaked at 21 days after BLM in the fibrosis-resistant male C3 and B6-11C3 mice compared with untreated counterparts ($P = 0.00128$ and $2.1 \times 10^{-7}$, respectively) (Fig. 5A). In contrast, BH activity in females of the fibrosis-resistant strains, C3 and B6-11C3, was not different at any time compared with their untreated counterparts ($P > 0.05$), although BH activity was significantly higher in the B6-11C3 strain than in female B6 mice ($P = 0.0012$) (Fig. 5B). Enzyme activity was not different at any time between untreated mice of the three strains.

**DISCUSSION**

Using linkage data and phenotyping congenic strains, we narrowed the interval containing Blmpf1 to a region 2.7 cM long. Based on the heritability estimates, this is the major locus affecting BLM-induced fibrosis, as it accounts for ~40% of the genetic contribution to this trait. This genomic region

![Figure 2. Schematic of chromosome 17 for the congenic strains B6.AKR-H2b, fibrosis resistant (FR), C3.SW-H2a, fibrosis prone (FP), the fibrosis-prone (FP) reduced congenic strain C3H-H2b and a second reduced congenic strain that is fibrosis resistant, C3H.H2b. The H2b and H2c regions on each chromosome are indicated by the open and solid boxes respectively. The linkage map shows the location of the peak marker within the congenic region in the reduced congenic strain, C3H.SW-H2b/C3H.D17Mit175-D17Mit47.](https://academic.oup.com/hmg/article-abstract/11/16/1855/657238)

overlaps with other significant lung response QTLs, including susceptibility to asthma (14), and radiation-induced (15), ozone-induced (16) and particle-induced lung inflammation (2); the last three QTLs were identified using the same two inbred parental strains as the current study. The complete reversal of the phenotype in the two congenic strains by changing the H2 allele indicates that the MHC has a major role in influencing susceptibility to BLM-induced fibrosis. The implication of this same locus in other models of pulmonary fibrosis supports the existence of a ‘fibrotic’ gene (9,10). Our findings in an animal model parallel observations in humans that the MHC is involved in the etiology of pulmonary fibrosis as a part of systemic sclerosis (25) and silica-induced pulmonary fibrosis (26). Among the genes mapped to Blmpf1, tumor necrosis factor-alpha (TNF-$\alpha$) has been physiologically implicated in BLM-induced pulmonary fibrosis (27,28). Specifically, Piguet et al. (27) showed that TNF/lymphotoxin knockout mice developed less pulmonary fibrosis following BLM treatment than their wild-type littermate controls.

We localized the second QTL, Blmpf2, to an 18 cM region of chromosome 11 in male mice only and confirmed this locus in a chromosome substitution strain, C57BL/6J-11C3H1, in which chromosome 11 in the C57BL/6J strain was replaced with chromosome 11 from the C3Hf/Kam strain. This QTL region also overlaps with those identified in other linkage studies of lung disease, including fibrosis induced by ozone (16) or particle exposure (2) and asthma (14), although the linkage was only suggestive in these studies. The prior studies did not report evidence of this sex specificity or any significant interaction between the loci on chromosomes 11 and 17. However, our study cohort is far larger than those of previously published studies and so may have more power to detect interactions.

<table>
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<tr>
<th>Table 2. Mean fibrosis with genotypes at peak linkage markers for chromosomes 11 and 17: males</th>
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<tr>
<td>----------------------------------------</td>
</tr>
<tr>
<td>D11Mit272</td>
</tr>
<tr>
<td>B6B6</td>
</tr>
<tr>
<td>B6C3</td>
</tr>
<tr>
<td>C3C3</td>
</tr>
<tr>
<td>(6.27)</td>
</tr>
<tr>
<td>(1.51)</td>
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<tr>
<td>(1.60)</td>
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<td><em>Mean fibrosis ($\bar{X}$) (SD).</em></td>
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<th>Table 3. Mean fibrosis with genotypes at peak linkage markers for chromosomes 11 and 17: females</th>
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<td>B6C3</td>
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<tr>
<td>(4.99)</td>
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<tr>
<td>(3.70)</td>
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<tr>
<td><em>Mean fibrosis ($\bar{X}$) (SD).</em></td>
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</table>
C3H alleles at chromosome 11 avert pulmonary fibrosis in male B6-11C3 mice, despite the presence of the fibrosis-susceptible chromosome 17 B6 H2b allele, consistent with the linkage data. Furthermore, the functional studies of BH activity implicate this enzyme in modulation of expression of the phenotype. BH activity was consistent with the phenotype in the different strains; that is, it was increased in the fibrosis-resistant strains and decreased in the fibrosis-prone strains. These data support the putative interaction between the two loci and implicate BH as a candidate gene for Blmpf2. However, BH maps distal to the QTL peak on chromosome 11 at the border of the statistically defined linkage interval (National Center for Biotechnology Information (NCBI), Bethesda, Maryland www.ncbi.nlm.nih.gov). Conditioning on D11Mit320, the marker closest to the position of BH on our linkage map, reduces the LOD score over the remainder of the chromosome (LOD < 1.0). Thus linkage analysis cannot exclude the possibilities that there is more than one susceptibility locus in the linkage region on chromosome 11 or that BH may be one of these loci. Finally, BH functions as a MHC class I epitope processing protease (29,30) and this function provides a possible mechanism for the interaction of Blmpf1 (MHC) and Blmpf2 (MHC processing).

Other candidate genes for Blmpf2 include spar (secreted acidic cysteine-rich glycoprotein) and the IL-4 cytokine gene family cluster, which map to the region of linkage at the peak QTL marker, D11Mit272. Sparc is polymorphic between the parental strains of this study and is overexpressed in the lungs of B6 mice following BLM administration (31). Physiologically, spar has a role in fibroblast migration in pulmonary fibrosis (32).

Whether or not BH is a susceptibility locus for BLM-induced pulmonary fibrosis remains unclear, but the role of this enzyme in modulating this phenotype is well established. The correlation of increased BH activity with the non-fibrotic

Figure 3. Hematoxylin and eojin (H&E)-stained left lung sections from C3, B6, MHC congenic and C3H-H2b reduced congenic male mice following treatment with 100 mg/kg bleomycin, with percentage pulmonary fibrosis for each section shown. (A) C3Hf/Kam %PF = 0; (B) C57BL/6J, %PF = 12.4; (C) C3.SW-H2b/SnJ, %PF = 13.6; (D) B6.AKR-H2b, %PF = 0; and (E) C3H-H2b reduced congenic, %PF = 8.3.
The presence of this enzyme protects against BLM-induced tissue damage (24,33), indicating that metabolism plays a significant role in determining the effect of BLM on the lungs. Tissues with naturally low levels of this enzyme, e.g. skin and lung, are particularly sensitive to BLM (24,33). Furthermore, BH knockout mice are more susceptible to BLM-induced death than their wild-type littermates (33). Conversely, overexpressing BH in transgenic mice provided protection from BLM-induced lung fibrosis (34). However, the difference in BLM activity between males and females treated with BLM found by us indicates a sex specificity not previously reported which may have implications for the use of this and other drugs in the clinic.

The studies in female B6-11C3 mice showed that BH activity also varies according to sex, but the effects were smaller than in males and not sufficient to totally avert fibrosis. We attribute the small but significant amount of fibrosis in the female B6-11C3 mice to Blmpf1 in the B6 background strain that is the major susceptibility locus for BLM-induced lung fibrosis in females. Although it is likely that Blmpf1 and Blmpf2 influence multiple fibrotic phenotypes, the interaction and sex specificity identified in the present work may indicate the existence of a fibrotic pathway particularly induced by BLM.

From the prior inheritance study (12), the existence of an X-linked locus explaining ~19% of the variance in F1 males was proposed, although there was, paradoxically, little evidence for X linkage in F2 mice. In the current mapping study, no evidence of an X-linked locus was found. Our current findings rather suggest only the effects of epistatic interaction that is only present in males. The confirmation of a major gene at the MHC as an influence on susceptibility to BLM-induced pulmonary fibrosis, coupled with its implication in other mouse models of lung fibrosis, suggests that a common genetic factor influences this response. The localization of Blmpf1 to a 2.7 cM region within the MHC will facilitate causative gene identification. Finally, the identification of Blmpf2 and the sex specificity through which it interacts with Blmpf1 may help elucidate one mechanism of pulmonary fibrosis.

### MATERIALS AND METHODS

#### Mouse model of pulmonary fibrosis

We used our previously described model of BLM-induced fibrosis in these studies (12). Briefly, both male and female C3Hf/Kam (C3) (fibrosis-resistant) and C57BL/6J (B6) (fibrosis-prone) mice were bred and maintained in the specific pathogen-free animal colony of the Department of Experimental Radiation Oncology. All other mice were housed in microisolator cages in the specific pathogen-free room in the Department of Veterinary Medicine. Congenic mice (C3.SW-H2b/SnJ and B6.AKR-H2b) were purchased from Jackson Laboratories. Mice were treated at 8–10 weeks of age. The data set includes 211 F2 mice from the previous inheritance study (12). An additional 150 F2 mice were treated as before, and there were no differences in the fibrotic phenotype between study groups ($P = 0.79$). In all, 214 F2 males and 147 F2 females were genotyped for chromosomes 17 and 11. For the congenic studies, 46 congenic mice, 9 B6.AKR-H2b and 37 C3.SW-H2b/SnJ, and 96 inbred partner strain mice, 57 C3 and 39 B6, representing both sexes, were treated with BLM and phenotyped.

Lung damage was elicited by administering BLM through osmotic minipumps implanted subcutaneously, as described previously (12). Male mice received 100 mg/kg and females 125 mg/kg BLM.

#### Generation of chromosome substitution strain

We generated the C57BL/6J-11C3 (B6-11C3) strain by a 'speed congenic' strategy (23). Six generations of marker-directed backcrosses were followed by an intercross to fix the C3Hf/Kam-derived chromosome 11 on the C57BL/6J background. We confirmed the genotype of these mice with 132 markers polymorphic between C3 and B6 mice and spaced at regular intervals throughout the genome. For the phenotyping experiments, 25 female and 12 male B6-11C3 mice were treated with our standard dose of BLM, and the percentage pulmonary fibrosis (%PF) was determined as described below; these data were compared with those from a cohort of 24 female and 15 male B6 mice treated and assayed at the same times. We assessed BH activity in 9 male and 15 female BLM-treated B6-11C3 mice.

#### Generation of reduced congenic strain

We constructed a strain containing a reduced congenic region by breeding the congenic C3.SW-H2b (fibrosis-prone) strain and the C3Hf/Kam (fibrosis-resistant) strain. Briefly, we intercrossed the progeny of a C3.SW-H2b × C3Hf/Kam mating. We genotyped the offspring for two markers, D17Mit16 and D17Mit50, at the proximal and distal ends respectively of the C3.SW-H2b congenic region to identify mice carrying recombinations within the donor MHC region. These mice were mated to C3Hf/Kam mice to generate additional individuals with the recombinant MHC that were

### Table 4. Phenotypic data for C3, B6, MHC congenic, reduced congenic and chromosome substitution strains

<table>
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<tr>
<th>Stain</th>
<th>Male</th>
<th>Female</th>
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<th>Female</th>
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<td></td>
<td>n</td>
<td>Mean (SE)</td>
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<td>Mean (SE)</td>
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<td>C3Hf/Kam</td>
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<td>9.2 (2.1)</td>
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<td>C57BL/6L-11C3H</td>
<td>12</td>
<td>0.9 (0.2)</td>
<td>25</td>
<td>2.5 (0.5)</td>
</tr>
<tr>
<td>F2</td>
<td>14</td>
<td>1.6 (0.4)</td>
<td>9</td>
<td>3.9 (1.0)</td>
</tr>
</tbody>
</table>

b $P < 0.05$ versus B6.
then intercrossed to establish a new congenic line homozygous for the reduced donor MHC region. We determined the phenotype of these mice to BLM, and of two reduced strains produced, only one exhibited the fibrotic phenotype. We generated experimental mice by brother–sister mating. The genotype of these mice was confirmed with 32 markers on chromosome 17. We named this strain C3H.SW-H2\textsuperscript{b}. C3H-D17Mit175–D17Mit47 (C3H-H2\textsuperscript{b} reduced congenic).

In total, 19 C3H-H2\textsuperscript{b} reduced congenic mice (12 males and 9 females) received BLM according to our standard protocol as described above, and the lungs were phenotyped at sacrifice as described below. We compared these data to a cohort of 10 male and 15 female BLM-treated mice assayed at the same times.

Histology and fibrosis scoring

At autopsy, the lungs were removed and the single left lobe of each mouse was perfused with 10% neutral buffered formalin and stained with Masson’s trichrome to identify the site(s) of collagen deposition in the lung. The area of the fibrosing phenotype for each mouse was quantified with image analysis of histologic sections as described elsewhere (12). Specifically, the area of fibrosis in the left lung lobe was determined from a user-drawn region surrounding the fibrosis and compared to the area of the entire lobe to yield the percentage of pulmonary fibrosis (%PF) for individual mice.

Genotyping with microsatellite markers

DNA from F2 animals was prepared from liver samples collected at necropsy using a DNA extraction kit (Puregene Gentra Systems, Inc., Minneapolis, MN). F2 intercross progeny were genotyped by polymerase chain reaction (PCR) using mouse markers defined by Dietrich et al. (35), as previously described (12).

QTL analysis

The data were analyzed using maximum likelihood methods in MapManager software (36) to identify loci that influence susceptibility to BLM-induced pulmonary fibrosis. MapManager was also used to compare recessive, dominant and additive models for the action of an identified QTL. The program was run initially to find putative QTLs, and successively rerun conditioning on identified QTLs (on chromosomes 17 and 11) to identify all genomic areas with LOD scores exceeding 2.8. With the computed LOD scores, linkage was assessed using the standards proposed by Lander and Kruglyak (37). The region where the LOD score decreased by a value of 1 on either side of the peak defined the confidence limit of the position of each QTL (19). The likelihood ratio statistic (LRS) of MapManager was converted to a LOD score by dividing the LRS by 4.6 (\(\frac{2 \ln}{4.6}\)). To comply with the statistical assumptions in the mapping software, the phenotypic data were arcsin transformed to stabilize the variance and so provide a more normally distributed variable (38). To identify linkage standards for our non-normal distribution phenotype, we further investigated the existence of the suggested QTLs using a permutation test of Churchill and Doerge (39) with 10,000 replicates. We performed this simulation study using the phenotypic and marker data (154 markers) from all of the mice that had been
In this analysis, for the additive model, the heterozygote mean is the average of the homozygote means. For the dominant model, the heterozygotes are assumed to have the same mean as the C3H/C3H homozygotes. For the recessive model, the heterozygotes are assumed to have the same mean as the B6/B6 homozygotes. Tests were constructed to compare these different models by obtaining the difference in LOD scores between the general model and one of the three inheritance models described above. This difference was multiplied by 4.6 to convert it to a chi-squared variable. We then compared the value to the chi-square distribution having 1 degree of freedom to obtain a P-value for the tests of additive, dominant or recessive inheritance.

**Bleomycin hydrolase assay**

Post-microsomal 105 000 g supernatants from rinsed lung tissue were prepared as previously described (24). Protein content was determined and the remaining sample stored at −80°C until analyzed. BH activity was determined by measuring the rate of conversion of bleomycin A2 (BLM A2) to its metabolite desamido-bleomycin A2 (dBLM) as described (24,41). Briefly, aliquots of prepared lung homogenates were incubated (37°C for 3 h) with 50 μg BLM A2 and 0.1 M sodium phosphate buffer (pH 7.2) in a final volume of 500 μl. Cold methanol (0.5 ml) was used to stop the reaction, and samples were centrifuged (15 000 g for 10 min). Aliquots (0.1 to 0.15 ml) of the supernatant were analyzed for dBLM content by reverse-phase HPLC. Separation of BLM and dBLM A2 was achieved using a Rainin Microsorb C8 column (Woburn, MA, USA, 4.6 × 250 mm) with an isocratic mobile phase (pH 5.0) consisting of CH3OH–CH3CN · H2O–CH3COOH (160 : 72 : 760 : 8) as well as 2 mM heptane sulfonic acid and 25 mM triethylamine. Relative fluorescence of BLM A2 and dBLM A2 was measured with an excitation wavelength of 297 nm and an emission wavelength of 355 nm. Peak identities were confirmed by co-injecting BLM A2 and dBLM A2 standards with the reaction mixtures. Preliminary studies were conducted to ensure that product formation was linear. The assay was validated with respect to reproducibility, accuracy and precision (data not shown).

Cohorts of male and female B6 (26 and 13, respectively), C3 (26 and 38, respectively) and B6-11C3 (6 males and 12 females) mice were treated with BLM and BH activity assayed in three mice each at weekly intervals for 4 weeks. Enzyme activity was assessed in separate cohorts of untreated male and female B6 (16 and 13 respectively) and C3 (16 and 16, respectively) mice assayed at the same time as the treated mice. Mean values and SDs were calculated and plotted as a function of time for each strain. Student’s t-test was used to determine the significance of differences.

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REFERENCES