Two genes abrogate the inhibition of murine hepatocarcinogenesis by ovarian hormones

(linkage analysis/sexual dimorphism)

THERESEx M. POOLE AND NORMAN R. DRINKWATER*

McArthur Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, WI 53706

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ABSTRACT Hormonal and genetic factors strongly influence the susceptibility of inbred mice to hepatocarcinogenesis. Female C57BR/cdJ (BR) mice are extremely susceptible to liver tumor induction relative to other strains because they are genetically insensitive to the inhibition of hepatocarcinogenesis by ovarian hormones. To determine the genetic basis for the sensitivity of BR mice relative to resistant C57BL/6J (B6) mice, we treated 12-day-old B6BRF1 × B6 and B6BRF1 × B6B6RF1 (F2) males with N,N-diethylnitrosamine (0.1 µmol/g of body weight) and enumerated liver tumors at 32 weeks of age in males and at 50 weeks in females. Genomic DNA samples from backcross and F2 mice were analyzed for 70 informative simple sequence length polymorphism markers. Genetic markers on chromosome 17 (D17Mit21) and chromosome 1 (D1Mit33) cosegregated with high tumor multiplicity in both sexes. Together, these loci (designated Hcfl and Hcf2 (Hepatocarcinogenesis in females), respectively) account for virtually all of the difference in sensitivity between BR and B6 mice. The Hcfl locus accounts for a majority of the higher susceptibility of BR mice of both sexes. Backcross female mice heterozygous at both loci (33 ± 23 tumors per mouse) and at Hcfl only (17 ± 18) were 15- and 8-fold more sensitive, respectively, than mice homozygous for the B6 alleles at Hcfl and Hcf2 (2.2 ± 3.9). In backcross male mice, the double heterozygotes (35 ± 22) and Hcfl heterozygotes (28 ± 12) were 5.4- and 4.3-fold more sensitive than mice homozygous for B6 alleles at both loci (6.5 ± 5.4).

The risk for liver cancer development is sex-dependent in both humans and mice. The incidence of primary hepatocellular carcinoma (HCC) is 2- to 5-fold higher in men than in women (1–4). At present, it is not known whether this sex difference results from differences in the exposure of men and women to risk factors, such as hepatitis B virus (3, 5), aflatoxin B1 (6), alcohol consumption (1), or from hormonal effects on the pathogenesis of liver cancer (4–6). Sensitivity to hepatocarcinogenesis in mice is highly sexually dimorphic. Male mice have a higher incidence of spontaneous liver tumors than females (7, 8) and are more susceptible to hepatocarcinogenesis following perinatal treatment with a variety of carcinogens (9–11). The sexual dimorphism in murine hepatocarcinogenesis results from the contrasting effects of androgens and ovarian hormones on liver tumor induction; androgens promote hepatocarcinogenesis whereas ovarian hormones inhibit the development of liver tumors (11–13). Thus, castration of male mice results in a decreased yield of liver tumors, whereas ovariec- tomy of females increases liver tumor multiplicity relative to intact animals (12–14).

One exception to this strong, sex-dependent effect on liver tumor induction in mice is the response of the C57BR/cdJ (BR) inbred strain. Whereas BR males are intermediate among inbred strains in sensitivity to hepatocarcinogenesis, the mean tumor multiplicity following perinatal carcinogen treatment in BR females is 15–30 times higher than that observed in the females of other inbred strains (10). The unusual susceptibility of the BR female is characterized by its insensitivity to the suppressing effects of ovarian hormones. In most strains, ovariec-tomized mice are significantly more susceptible than intact females to liver tumor induction (11, 12) but ovariec-tomized and intact BR females have similar mean tumor multiplicities (14, 15). In contrast, the responsiveness of BR male mice to the promotion of hepatocarcinogenesis by androgens is similar to that observed in other strains (14, 15).

There are large differences among inbred strains in the sensitivity of male mice to hepatocarcinogenesis (7, 9, 10). Analysis of segregating crosses between strains that vary widely in their susceptibility provides a means for mapping genes that contribute to the sensitivity to liver tumor induction. Our laboratory and others have been successful in identifying loci that influence the development of liver tumors in the males of sensitive strains, such as C3H/HeJ (C3H) and DBA/2J (D2), using linkage analysis. Dragani and coworkers (16, 17) mapped several Hepatocarcinogen sensitivity (Hcs) loci in crosses between C3H mice and various resistant strains. We have analyzed segregating crosses between C3H and B6 mice and demonstrated that a locus in the distal region of chromosome 1 is largely responsible for the difference in mean tumor multiplicity between these two strains (18, 19). This Hcs locus is thought to affect the growth of preneoplastic hepatic foci (20). Two Hepatocarcinogen resistance (Her) genes were mapped to chromosomes 4 and 10 in crosses between the D2 and B6 strains (21). Based on biological studies of hepatic focus growth, it is postulated that these loci may also affect the growth rate and/or formation of preneoplastic lesions (20, 22).

While the range of variation in susceptibility among inbred strains is narrower for female than for male mice, the rank orders of susceptibilities within the sexes are similar (10). This observation is consistent with a sex-independent effect for the susceptibility loci described above. Understanding the unique responsiveness of female BR mice should provide insight into the causes of the relative resistance of the female mice of most inbred mouse strains. Analysis of the segregation of genes in crosses between sensitive BR mice and a resistant strain may result in the identification of genes that counteract the suppressing effects of ovarian hormones in BR females. In addition, because there are similar sex differences in the susceptibility to liver tumor development in humans, genes identified in these studies may provide insight into the determinants of the sex-dependent risk for HCC in humans.

Abbreviations: B6 mice, C57BL/6J mice; BR mice, C57BR/cdJ mice; DEN, N,N-diethylnitrosamine; SSLP, simple sequence length polymorphism; HCC, hepatocellular carcinoma; lod, logarithm of odds; cm, centimorgan.

*To whom reprint requests should be addressed. email: drinkwater@oncology.wisc.edu.

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We performed linkage analysis on crosses between closely related BR and resistant B6 mice and mapped two genes that account for nearly all of the difference in sensitivity in both males and females of these strains. Although these genes act additively to increase the susceptibility of both sexes to liver tumor induction, the magnitudes of their effects are larger in female mice than in male mice.

METHODS

Mice and Liver Tumor Induction. Male and female C57BL/6J and C57BR/cdJ mice were bred from stocks purchased from The Jackson Laboratory. Mice were housed in plastic cages on corncob bedding (Bed O’Cobs, Anderson Cob Division, Maumee, OH) and fed Wayne Breeder Blox (Continental Grain Company, Chicago) and acidified tap water ad libitum. Mice were inspected daily and weighed monthly. Mice from the following crosses were used for tumor induction studies: B6, BR, (B6 × BR)F1, (BR × B6)F1, B6BRF1 × B6, B6BRF1 × B6B6, BRF1 × B6 and B6BRF1 × B6BRF1 (F2). Male and female mice (all 12 days old) from each cross were treated with N,N-diethylnitrosamine (DEN) (0.1 μmol/g of body weight) dissolved in sterile triacetanoin (0.01 ml/g) (Pfaltz & Bauer). Mice were killed by CO2 asphyxiation at 32 weeks (males) or 50 weeks (females) of age and tumors larger than 1 mm on the surface of the liver were enumerated. Spleens were removed and frozen on dry ice as a source of genomic DNA. A random sample of tumors was excised, fixed in formalin, and histologically analyzed after staining with hematoxylin and eosin.

Genotypic Analysis. DNA was isolated from spleens using a standard proteinase K-phenol extraction protocol (23). Of ~300 simple sequence length polymorphism (SSLP) markers (24) tested for polymorphism between B6 and BR, 70 were found to be informative. These markers had an average spacing of about 20 centimorgans (cMs) and allowed coverage of about 85% of the total mouse genome for linkage analysis. Polymerase chain reaction (PCR) was performed using 50–100 ng of genomic DNA in a mixture containing the following reagents (final concentrations): 0.17 μM each primer, 60 μM each dNTP, 10 mM Tris (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 0.1 mg/ml gelatin, 0.5 units Taq polymerase (Boehringer Mannheim). A 50-cycle program of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec was used in a DNA thermal cycle (Perkin-Elmer/Cetus). A 20-μl sample was loaded onto a 7% polyacrylamide gel and electrophoresed. The gel was stained with ethidium bromide, photographed under UV light, and scored for the presence of B6- and BR-specific alleles.

Linkage Analysis. B6BRF1 × B6 male and B6BRF1 × B6 female backcross mice (57 each) were scored for their genotypes at 70 SSLP loci. Male (B6BRF1 × B6BRF1)F2 (68) and female (B6BRF1 × B6BRF1)F2 (65) mice were genotyped for the SSLP markers on chromosomes 1 and 17. The SSLP markers used in this study were: chromosome 1, D1Mit1, -5, -10, -17, -20, -33, -88, -89; chromosome 2, D2Mit35, -51, -74; chromosome 3, D3Mit14, -25, -40; chromosome 4, D4Mit33, -39, -41; chromosome 5, D5Mit7, -10, -14, -43, -48, -50; chromosome 6, D6Mit1, -10, -33, -44; chromosome 7, D7Mit12, -44, -57, D7Nd4; chromosome 8, D8Mit11, -16; chromosome 9, D9Mit6, -8, -42, -53, D9Nd2; chromosome 10, D10Mit11, -51, -70; chromosome 11, D11Mit4; chromosome 12, D12Mit4, -5, -63, D12Nd2; chromosome 13, D13Mit3, -18; chromosome 14, D14Mit28, -30, -36, -42; chromosome 15, D15Mit13, -16, -31; chromosome 16, D16Mit4, -5, -7; chromosome 17, D17Mit10, -16, -21, -23, -42; chromosome 18, D18Mit9, -10, -33; and chromosome 19, D19Mit16, -42.

Because liver tumor multiplicity data do not follow the normal distribution (25), we used a nonparametric approach described by Kruglyak and Lander (26) to assess linkage between the marker loci and the quantitative trait loci that determine liver tumor multiplicity. For backcross mice, the data for each marker were analyzed independently for female and male mice using the Wilcoxon rank sum test (27) to obtain the test statistic, Z. The significance level for the test of the hypothesis that the marker locus was linked to a quantitative trait locus was determined using the intermediate map approximation described by Kruglyak and Lander (26). The threshold for a genome-wide significance level of P = 0.05 for our study is 3.45, nearly identical to that for the conservative sparse map approximation; the corresponding value for the dense map case is 3.85. Equivalent lod (logarithm of odds) scores (26), lodw, were estimated as lodw = 0.5 (log10 e) (Z2).

An analogous approach using the Jonckheere-Terpstra test (27) was used to analyze the data for intercross mice. The significance level for the overall hypothesis of linkage was determined by combining the results of the four sex × cross groups using a method described by Fisher (28). Briefly, if F is the observed significance level for group i, the value (−2 x ln Pi) is distributed as a χ2 variable with 2n degrees of freedom. The estimated significance level was then adjusted to the dense map approximation of Kruglyak and Lander (26).

We also analyzed the backcross and intercross data jointly using log-linear models for the susceptibility phenotype, T, of the number of tumors per mouse, of the following form:

\[
\log(T + 1) = b_0 + a_{Hcf1}T_1 + a_{Hcf2}T_2 + d_{Hcf1}T_1 + d_{Hcf2}T_2 + e_1T_5 + e_2T_6 + e_3T_7 + e_4T_8 + \epsilon
\]

The parameters for the additive effect, aHcf1 and aHcf2, represent the effect of carrying a single BR allele at Hcf1 and Hcf2, respectively, whereas the dominance components, dHcf1 and dHcf2 denote the additional susceptibility conferred by homozygosity for the BR allele at the two loci. The parameters e1 to e3 take into account the interactions between the two loci, with i and j indicating the number of BR alleles at Hcf1 and Hcf2, respectively. Three models were analyzed: (i) a full interaction model in which four eij were individually specified; (ii) a simpler interaction model in which all of the eij were assumed to be identical; and (iii) a simple additive model with the eij = 0. The xij are indicator variables that depend on the genotype of the animal at the markers nearest Hcf1 (D17Mit21) and Hcf2 (D11Mit33); e is the error term.

RESULTS

Sensitivities of Segregating Crosses to Hepatocarcinogenesis. The mean tumor multiplicities of the parental strains, F1 hybrids, and the segregating crosses are presented in Table 1. Because liver tumors arise earlier in male mice than in females, animals were sacrificed at 32 and 50 weeks of age for males and females, respectively. The yield of liver tumors in female BR

<table>
<thead>
<tr>
<th>Cross</th>
<th>Females No. of mice</th>
<th>Female Mean tumor multiplicity (sd)</th>
<th>Males No. of mice</th>
<th>Male Mean tumor multiplicity (sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>33</td>
<td>4.5 (5.5)</td>
<td>32</td>
<td>6.2 (8.2)</td>
</tr>
<tr>
<td>BR</td>
<td>35</td>
<td>66 (41)</td>
<td>32</td>
<td>36 (23)</td>
</tr>
<tr>
<td>B6BRF1</td>
<td>31</td>
<td>60 (32)</td>
<td>30</td>
<td>43 (22)</td>
</tr>
<tr>
<td>B6BRF1 × B6</td>
<td>57</td>
<td>15 (19)</td>
<td>57</td>
<td>20 (19)</td>
</tr>
<tr>
<td>B6BRF1 × BR</td>
<td>57</td>
<td>52 (30)</td>
<td>47</td>
<td>62 (33)</td>
</tr>
<tr>
<td>B6BRF2</td>
<td>65</td>
<td>22 (21)</td>
<td>68</td>
<td>39 (33)</td>
</tr>
</tbody>
</table>

Male and female mice (all 12 days old) were treated with DEN (0.1 μmol/g of body weight) and liver tumors were enumerated at 32 weeks of age in males and 50 weeks in females. Values are mean [standard deviation (sd)].
mice (66 ± 4i SD) was 15-fold higher than that in B6 females (4.5 ± 5.5), whereas tumor multiplicity in BR males (36 ± 23) was 5.8-fold higher than in B6 males (6.2 ± 8.2). Male and female B6BRF1 mice (female, 60 ± 32; male, 43 ± 22) were similar in susceptibility to BR mice, indicating that any major genes controlling sensitivity were expressed in at least a semidominant manner. The reciprocal F1 cross (BR × B6) was also highly susceptible to liver tumor induction (female, 36 ± 22; male, 59 ± 31), indicating that X-linked genes did not play a major role in controlling sensitivity to hepatocarcinogenesis in male mice.

The distributions of tumor multiplicities for backcross and intercross mice of both sexes (Fig. 1) were bimodal, indicating that a small number of segregating loci accounted for the susceptibility to tumor induction. This conclusion was further supported by Wright’s method for estimating the number of effective loci from the variances of the backcrosses and intercrosses and the means and variances of the parental and F1 hybrid mice (29). After transformation of the tumor multiplicity data to remove the dependence of the variance on the mean (30), Wright’s method yielded estimates of approximately one and two loci for the intercross and backcross, respectively.

**Linkage Analysis in Backcross Mice.** All of the autosomes were scanned for quantitative trait loci that influence susceptibility to liver tumor induction by genotypic analysis of 70 SSLP markers in 57 male and 57 female backcross mice. In this initial screen, markers on chromosome 17 cosegregated with the susceptible phenotype in both male and female mice (Table 2). The strongest association was for D17Mit21, which provided significant linkage statistics, Zsc, of 3.55 (P < 0.04, genome-wide significance level) and 4.75 (2 < 2 × 10–4), for females and males, respectively. We designated the susceptibility locus linked to D17Mit21 as Hcf1. The only other chromosomal region that yielded a value of Zsc that exceeded 2.0 was found on chromosome 1, near D1Mit10 (Zsc = 2.63) for the male backcross mice and near D1Mit33 (Zsc = 2.29) for the female backcross mice. However, the distribution of tumor multiplicities in backcross female mice for animals that were heterozygous at D17Mit21 was clearly bimodal (data not shown), providing a strong indication that a second locus contributed to the susceptibility of heterozygous females. We reanalyzed the genotypic data for those females that were heterozygous at D17Mit21 and observed that only markers on chromosome 1 provided a value of Zsc exceeding 2.0. As observed for the males, the best association was for the marker D1Mit10 (Zsc = 2.89 for D1Mit21 heterozygotes; data not shown). Although the linkage of susceptibility to chromosome 1 was not significant for either male mice or the D17Mit21 heterozygous females, combining the results for these two groups of backcross mice provided a strong indication that this region contained a susceptibility locus (P < 0.01). Accordingly, we tentatively designated the liver tumor susceptibility locus linked to D1Mit10 and D1Mit33 as Hcf2.

**Analysis of Intercross Mice for Confirmation of Putative Liver Tumor Susceptibility Loci.** Based on the results for the backcross mice, male and female F2 mice were genotyped for DNA markers on chromosomes 17 and 1. Analysis of the intercross mice confirmed the conclusions drawn in the backcrosses; inheritance of BR alleles for markers on chromosome 17 correlated well with sensitivity in both sexes. Significant linkage of susceptibility to D17Mit21 was observed in both sexes, with Zsc values of 4.85 (P < 2 × 10–4, genome-wide significance level) and 3.59 (P = 0.03) for females and males, respectively. In males, the adjacent marker (D1Mit16), ~10 cM proximal to D1Mit21, provided the strongest evidence of linkage (P = 0.012). In contrast to the backcross analysis, significant linkage of liver tumor susceptibility to markers on chromosome 1 was detected in the F2 mice. In females, the marker D1Mit10 yielded a significant result (Zsc = 3.89, P = 0.02), while in males, a marker ~20 cM distal, D1Mit33, provided the strongest evidence of linkage (Zsc = 3.93, P < 0.01).

We combined the analyses of the four sex × cross groups using an approach described by Fisher (28) and estimated an equivalent lod score as described by Kruglyak and Lander (26) (Table 2). Taken together, analyses of both crosses provided extremely strong evidence for a liver tumor susceptibility locus, designated Hcf1, on chromosome 17 near D1Mit21 (P < 10–10, lod = 15.5). A second locus, Hcf2, was also identified on chromosome 1 near D1Mit33 (P < 0.005, lod = 6.7).

In general, the recombination distances that we observed for backcross and intercross mice for the markers on chromosomes 1 and 17 were similar to those indicated by the most recently published maps for the mouse genome (31, 32). However, we observed greater than expected frequencies of recombination for the markers on proximal chromosome 17. The published map for this region is as follows: D17Mit23 is 1.8 cM from D1Mit16 which is 0.4 cM from D1Mit21, whereas we observed distances of 13.9 cM (95% confidence interval, 10.5–17.8) and 7.3 cM (5.1–12.2), respectively.

**Resolution of the Distributions of Tumor Multiplicity in Segregating Crosses According to the Genotype at Hcf1 and Hcf2.** We compared the mean tumor multiplicities for backcross mice with those for BB6F1 hybrid mice because in both instances inheritance of BR alleles is through the mother. A large fraction of the difference in susceptibility between BR and B6 mice can be accounted for by the Hcf loci. In the females the proportion of the phenotype resulting from the BR alleles at these two loci is ~90%, and in the males it is ~60%. The mean tumor multiplicities for the backcross mice heterozygous for both loci (33 and 35 tumors per mouse in females and males, respectively) are similar to the means in the BB6F1 hybrid (36 and 59 tumors per mouse in females and males, respectively) indicating that these are the only major loci contributing to the tumor yield in BR mice.

Analysis of each genotypic class at the Hcf loci in the BB6F1 × B6 backcross allowed us to assign gene interactions and the relative contributions of the two genes to the sensitivity of BR mice to hepatocarcinogenesis. In females, these genes appear to interact in an additive manner (Table 3) as indicated by the fact that the backcross mice heterozygous for BR and B6 alleles at both chromosome 17 and chromosome 1 have a mean tumor yield that is 15 times higher than that in mice homozygous for B6 alleles at both loci. The mice heterozygous for chromosome 17 markers only or chromosome 1 markers only were 7.7- and 4.0-fold more sensitive, respectively, than...
Table 2. Linkage of DNA markers to susceptibility to liver tumor induction in segregating crosses

| DNA marker | Position, cM | Female: Zm (P value) | Male: Zm (P value) | Combined results
|------------|-------------|---------------------|------------------|-----------------
|            | Backcross   | Intercross          | Backcross        | Intercross     | $-2 \Sigma n P$ | lodm |
| Chromosome 17 |
| D17Mit23   | 16.4        | 3.42 (0.059)        | 2.57             | 4.42 (0.001)   | 3.32 (0.08)    | 61.0 ($6 \times 10^{-7}$) | 10.6 |
| D17Mit16   | 18.2        | 3.28 (0.013)        | 3.15 (0.15)      | 4.43 (0.001)   | 3.84 (0.012)   | 71.2 ($10^{-8}$) | 12.7 |
| D17Mit21   | 18.6        | 3.55 (0.036)        | 4.85 (1.20$x10^{-4}$) | 4.75 (2.0$x10^{-4}$) | 3.59 (0.03)    | 85.2 ($10^{-11}$) | 15.5 |
| D17Mit10   | 25.1        | 3.07 (0.2)          | 4.37 (0.001)     | 4.00 (0.006)   | 3.69 (0.02)    | 71.1 ($10^{-8}$) | 12.6 |
| D17Mit42   | 47.4        | 2.34                | 1.84             | 0.60           | 0.98           | 16.7                          | 2.2  |
| Chromosome 8 |
| D1Mit1     | 13.7        | 0.08                | 0.15             | 0.98           | 0.54           | 3.7                            | 0.3  |
| D1Mit5     | 32.8        | 1.20                | 2.97             | 1.07           | 2.54           | 26.1                          | 3.9  |
| D1Mit9     | 36.9        | 1.87                | 3.48 (0.047)     | 1.38           | 2.16           | 31.3 (0.13)                  | 4.8  |
| D1Mit10    | 56.6        | 0.78                | 3.69 (0.02)      | 2.63           | 2.26           | 35.5 (0.025)                 | 5.7  |
| D1Mit33    | 81.6        | 2.29                | 2.98             | 1.20           | 3.93 (0.008)   | 41.0 (0.0027)               | 6.7  |
| D1Mit17    | 106.3       | 2.41                | 0.20             | 0.74           | 0.69           | 11.6 (0.17)                 | 1.5  |

Backcross (B6BRF1 × B6) and intercross (B6BRF2) mice were injected with DEN (0.1 μmol/g of body weight) at 12 days of age. Liver tumors were counted at 32 weeks (males) or 50 weeks (females) and spleen DNA from each mouse was analyzed for SSLP genetic markers as described.

Chromosomal position is given in cM from the centromere (31, 32).

Linkage of the indicated marker to a locus conferring susceptibility to liver tumor induction was assessed using the nonparametric approach described by Kruglyak and Lander (26). The test statistic, $Z_m$, was computed using the Wilcoxon rank sum test for the backcross mice and the Jonckheere-Terpstra test for intercross mice. The significance level, given in parentheses, was determined for the intermediate map case, described by Kruglyak and Lander (26), for the collection of 70 markers analyzed in our study.

Values for the four sex × cross groups were combined using the method described by Fisher (28); the value of $-2 \Sigma nP$ follows a $x^2$ distribution with 8 degrees of freedom. The significance level was adjusted by the factor (C + 2GT$^2$) as described by Kruglyak and Lander (26) for the dense map case and the equivalent lod score was estimated as described in the same reference.

Table 3. Genetic interactions between the two susceptibility loci, Hcf1 and Hcf2, in B6BRF1 × B6 male and female mice treated with DEN

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>No. of mice (%)</th>
<th>Mean liver tumor multiplicity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17Mit21</td>
<td>D1Mit33</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H H</td>
<td>13 (24)</td>
<td>33 (23)</td>
</tr>
<tr>
<td>H B</td>
<td>18 (33)</td>
<td>17 (18)</td>
</tr>
<tr>
<td>B H</td>
<td>11 (20)</td>
<td>8.9 (7.1)</td>
</tr>
<tr>
<td>B B</td>
<td>13 (24)</td>
<td>2.2 (3.9)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H H</td>
<td>15 (28)</td>
<td>35 (22)</td>
</tr>
<tr>
<td>H B</td>
<td>8 (15)</td>
<td>12 (14)</td>
</tr>
<tr>
<td>B B</td>
<td>16 (30)</td>
<td>6.5 (5.4)</td>
</tr>
</tbody>
</table>

Mice (12 day old) were injected with DEN (0.1 μmol/g of body weight); liver tumors were counted at 32 weeks of age (males) and 50 weeks of age (females).

The mice were classified according to their genotypes at the markers closest to Hcf1 (D17Mit21) and Hcf2 (D1Mit33): H, heterozygous for BR and B6 alleles; B, homozygous for B6 alleles.

†Values are mean (SD).

additive and dominance components for each locus (Table 4). This model accounted for 85–90% of the variance in tumor multiplicity in both males and females; inclusion of the possibility of interaction effects for the two loci did not improve significantly the fit of the model to the data ($P > 0.5$ for both males and females). In females, mice heterozygous for BR alleles at both Hcf1 and Hcf2 were estimated to be ∼12-fold more susceptible to liver tumor induction than mice homozygous for B6 alleles at both loci. When compared with the doubly homozygous B6 mice, the Hcf1BR/BR Hcf2BR/BR genotype resulted in a 22-fold increase in tumor multiplicity, with Hcf1 and Hcf2 accounting for 7.0- and 3.2-fold increased susceptibility, respectively. In male mice, heterozygosity and homozygosity for the BR alleles at both loci resulted in 4.8- and 17-fold increases in sensitivity relative to the Hcf1B6/B6 Hcf2B6/B6 genotype and the two Hcf genes made similar contributions to the phenotype.

**Histological Analysis.** Sections from 235 formalin-fixed tumors were analyzed to determine the stage of progression of

Table 4. Analysis of segregation of susceptibility to liver tumor induction in backcross and intercross mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>exp(b0) (tumors/mouse)</td>
<td>1.8 ± 0.4*</td>
<td>5.4 ± 0.9</td>
</tr>
<tr>
<td>exp(aHcf1) (fold increase)</td>
<td>5.4 ± 1.1</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>exp(dHcf1) (fold increase)</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>exp(aHcf2) (fold increase)</td>
<td>2.3 ± 0.5</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>exp(dHcf2) (fold increase)</td>
<td>1.4 ± 0.5</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.85</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Tumor multiplicity data from backcross (B6BRF1 × B6) and intercross (B6BRF2) mice were analyzed using a log-linear model as described. The parameters denote the following: exp(b0), the expected number of tumors/mouse for animals homozygous for B6 alleles at Hcf1 and Hcf2; exp(aHcf1) and exp(aHcf2), the fold-increase in tumor multiplicity resulting from heterozygosity at the appropriate locus; exp(dHcf1) and exp(dHcf2), the fold-increase in tumor multiplicity resulting from the addition of a second BR allele at Hcf1 and Hcf2, respectively (i.e., the Hcf1BR/BR Hcf2BR/BR genotype would be expected to have [exp(aHcf1) × exp(dHcf1)]-fold more tumors than mice homozygous for the B6 allele); $R^2$, the fraction of the variance in susceptibility accounted for by the model.

*Values are estimated parameter ± SD.
the tumors. Of these, 164 (70%) tumors were hepatocellular adenomas, 35 (15%) were HCC, and 36 (15%) were mixed adenoma and carcinoma. Of the 71 HCC, 58 (85%) were found in female mice, but the later sacrifice time for females could account for the presence of more advanced tumors in this group.

DISCUSSION

Two loci account for a large fraction of the variation in sensitivity in both females and males derived from crosses between BR and B6 mice. These loci have been mapped to chromosomes 17 and 1 and have been designated Hcf1 and Hcf2, respectively, for Hepatocarcinogenesis in females because of the unique female susceptibility of BR mice. Interestingly, the two genes confer a high susceptibility to liver tumor induction in both sexes. This result may indicate that the genes identified in these crosses are part of a general tumor induction pathway that is not influenced by the normally suppressive effects of the female hormonal environment (14).

The use of two closely related strains of mice may have provided a less complex genetic model in which to segregate susceptibility genes. The B6 and BR lines were established from two siblings; ~25% of their genes are identical by descent (33, 34). The level of polymorphism between B6 and BR, as measured by published enzyme polymorphisms (33) and by our experience with the SSLP markers, is ~23% compared with the 40–54% polymorphism between a randomly chosen pair of inbred strains of mice (35). Although this common genetic background of BR and B6 mice originally presented an obstacle to mapping genes in crosses because of the low level of polymorphism, the SSLP markers (24, 36) are distributed throughout the genome at a sufficiently high density to allow coverage of most of the genome for linkage. In fact, the analysis of the segregation of genes in any two closely related strains should be more likely to result in the identification of only a few loci that account for a majority of the phenotype of interest.

The Hcf genes may be involved in regulating the growth of preneoplastic lesions in the liver. Hepatic foci in the livers of male and female BR mice have similar rates of growth. In females, the Hcf genes may serve to increase the growth rate of hepatic foci because preneoplastic foci grow more rapidly in BR females than in B6 or C3H females (15, 20). However, the focus growth rates are similar in BR and B6 males, indicating that the Hcf genes must also influence other properties of liver tumor formation (15). The number of foci in the livers of BR males is ~5-fold higher than that in B6 males and the livers of BR females contain about twice as many hepatic foci as those in B6 females (15). Thus, it is likely that at least one of the Hcf genes affects the process of focus formation. It has been demonstrated that a high proportion of tumors from BR males (37) harbor mutations in the Hras1 gene, whereas only a small fraction of tumors in B6 males (37, 38) contain mutations in this proto-oncogene. Thus, one or both of the Hcf genes may promote the outgrowth of cells with activating Hras1 mutations.

As discussed above, the Hcs gene identified in crosses between C3H and B6 mice has also been mapped to chromosome 1 (19). We have shown that hepatic foci are numerous in C3H male mice (20) and that a high percentage of tumors from the livers of C3H males harbor mutations in Hras1 (37, 38). Since Hcf2 has been localized to the same region of chromosome 1 as Hcs, it is tempting to speculate that they identify the same gene. Although Hcs accounts for a majority of the difference in sensitivity between C3H and B6 male mice, the genetics of susceptibility in C3H mice is very complex; several genes contribute to the high sensitivity of this strain (16–19). However, in the crosses presented here, two loci account for virtually all of the difference in sensitivity. Therefore, if Hcf2 is identical to Hcs, then the BR mouse may provide a simpler model to study the effects of this susceptibility gene.

There are several candidates for the Hcf genes that have been mapped to the appropriate region of mouse chromosome 17 or chromosome 1. A number of genes located on chromosome 17 are regulated in a sex-specific manner. One of these genes, Sfp, a component of complement, is expressed only in the males of some strains (39). In addition, Slp also regulates the sexually dimorphic expression of Cyp21a1 (40, 41), which is located near Sfp (42), and may possibly regulate the expression of other nearby genes. Other genes in the chromosome 17 interval that play roles in various signal transduction pathways include Pdk2, a retinoid X receptor that is involved in vertebrate development and cell growth (43, 44), Hsp70, the 70-Kd heat shock protein that participates in steroid hormone receptor activation (45, 46), and Tnfa, b, and c, which encode tumor necrosis factor (47, 48). The Hcf1 interval also includes the murine major histocompatibility complex. Analysis of tumor development in a large number of H2 congenic lines has revealed that genes in this region influence susceptibility to the induction of lung and intestinal tumors (49, 50). Candidate genes for Hcf2 include Abll, a tyrosine kinase related to the Abelson proto-oncogene (51), and Rerg, the retinoid X receptor gamma (52). The construction of congenic mouse lines (31) for the regions of chromosomes 17 and 1 that contain the Hcf genes will allow both detailed physical mapping of the interval and positional cloning of the genes and will also be useful for studying the effects of each Hcf gene in isolation.

Genetic analysis of liver cancer risk in humans is complicated by many environmental factors including chronic hepatitis B virus infection, aflatoxin exposure, alcohol consumption, and tobacco usage (1, 3, 5, 6). The sex-dependent susceptibility to hepatocarcinogenesis is consistent with the mouse model of hormonally regulated hepatocarcinogenesis. Thus, identification of genes that affect sex-dependent liver tumor induction in mice may provide important clues about tumor formation in humans.

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