Predominant modifier of extreme liver cancer susceptibility in C57BR/cdJ female mice localized to 6 Mb on chromosome 17

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Sex hormones influence the susceptibility of inbred mice to liver cancer. C57BR/cdJ (BR) females are extremely susceptible to spontaneous and chemically induced liver tumors, in part due to a lack of protection against hepatocarcinogenesis normally offered by ovarian hormones. BR males are also moderately susceptible, and the susceptibility of both sexes of BR mice to liver tumors induced with *N,N*-diethylnitrosamine relative to the resistant C57BL/6J (B6) strain is caused by two loci designated *Hcf1* and *Hcf2* (hepatocarcinogenesis in females) located on chromosomes 17 and 1, respectively. The *Hcf1* locus on chromosome 17 is the predominant modifier of liver cancer in BR mice. To validate the existence of this locus and investigate its potential interaction with *Hcf2*, congenic mice for each region were generated. Homozygosity for the B6.BR(*D17Mit164-D17Mit2*) region resulted in a 4-fold increase in liver tumor multiplicity in females and a 4.5-fold increase in males compared with B6 controls. A series of 16 recombinants covering the entire congenic region was developed to further narrow the area containing *Hcf1*. Susceptible heterozygous recombinants demonstrated a 3- to 7-fold effect in females and a 1.5- to 2-fold effect in males compared with B6 siblings. The effect in susceptible lines completely recapitulated the susceptibility of heterozygous full-length chromosome 17 congenics and furthermore narrowed the location of the *Hcf1* locus to a single region of the chromosome from 30.05 to 35.83 Mb.

Introduction

Hepatocellular carcinoma is the fifth most common neoplasm worldwide and the third most common cause of cancer-related death, causing >500 000 deaths per year (1). The incidence of hepatocellular carcinoma is 2- to 5-fold higher in men than in women, and it is uncertain if this difference is caused solely by the differing hormonal environments or is also influenced by differences in exposure to risk factors (2,3). Known risk factors for liver cancer include hepatitis B or C virus infection, alcohol consumption and aflatoxin B_1 ingestion (4).

Similar to humans, male mice have a higher incidence than female mice of both spontaneous liver tumors (5,6) and liver tumors following perinatal treatment with a variety of carcinogens (7,8). The sexual dimorphism in murine hepatocarcinogenesis is due to the contrasting effects of sex hormones on liver tumor induction. Castrated male mice develop fewer liver tumors than intact males, whereas ovariectomized females develop more liver tumors than their intact counterparts (7,9–11). Furthermore, gonadectomy results in a similar incidence of liver tumors in B6C3F$_1$ males and females (7). These studies demonstrate

that androgens promote liver tumors, whereas ovarian hormones inhibit their development.

C57BR/cdJ (BR) male mice have an intermediate susceptibility to liver tumors compared with males of other inbred strains, whereas BR females are extremely susceptible, developing up to 50-fold more tumors than females of all other strains tested (8,11). This high susceptibility is cell autonomous (12) and is due, in part, to a loss of the ovarian hormones' protection from liver tumor development. As a result, in contrast to other strains, ovariectomy in BR females does not cause a significant change in liver tumor multiplicity after treatment with *N,N*-diethylnitrosamine (DEN) (11). The loss of protection seen in BR females is not due to a difference in the amount or affinity of estrogen receptor in the liver of BR females (13). BR females, therefore, offer a unique opportunity to investigate how ovarian hormones influence susceptibility to hepatocarcinogenesis.

Ovarian hormones may cause a decrease in the growth rate of preneoplastic and neoplastic lesions. Preneoplastic lesions in the mouse liver are indicated by an abnormal deficiency in glucose-6-phosphatase (14). These lesions have similar growth rates in C57BL/6J (B6) and C3H/HeJ (C3H) females, and the growth rates in these females are lower than in the corresponding males (11,15). In contrast, preneoplastic lesions grow more rapidly in BR females than in B6 females, and the growth rates in BR males and BR females are similar. Furthermore, ovariectomy increases the growth rate of preneoplastic lesions in B6, C3H and B6C3F$_1$ females, but not in BR females (7,11,16–18). Therefore, the high susceptibility of BR female mice to liver tumors may be due to the inability of their ovarian hormones to inhibit the growth of preneoplastic lesions in the liver (11).

A linkage analysis of crosses between the resistant B6 and sensitive BR strains identified just two hepatocarcinogenesis in females (*Hcf*) loci that are responsible for the majority of the difference in susceptibility to DEN in both males and females (19). *Hcf1* is located on chromosome 17 and *Hcf2* is found on chromosome 1. These two loci together accounted for ~85–90% of the susceptibility in the BR strain compared with B6. The *Hcf1* locus alone was responsible for roughly two-thirds of the effect. The Hepatocarcinogen sensitivity 7 (*Hcs7*) locus has previously been identified on chromosome 1 in a linkage analysis of crosses between the C3H and B6 strains (20). This locus accounted for ~85% of the ~20- to 50-fold increase in liver tumor multiplicity in the susceptible C3H males compared with resistant B6 males. The *Hcf2* locus from BR and the *Hcs7* locus in C3H may be identical (20).

Congenic lines for each *Hcf* locus from BR on a B6 background were generated and their susceptibilities to DEN-induced hepatocarcinogenesis were evaluated to verify the existence of these loci as well as to investigate any potential interaction. In addition, a series of 16 recombinant lines spanning the majority of chromosome 17 were bred and similarly evaluated to further reduce the region known to contain *Hcf1*, the locus responsible for the majority of the susceptibility of the BR strain. Finally, the variation among B6, BR and C3H mice was studied by analyzing genotypes at known single nucleotide polymorphisms (SNPs) and sequencing non-coding regions of genes to generate haplotype maps of the identified region.

Materials and methods

Mice

B6 and BR mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our facilities. Mice were housed in plastic cages on corncob

Abbreviations: B6, C57BL/6J; BR, C57BR/cdJ; C3H, C3H/HeJ; DEN, *N,N*-diethylnitrosamine; IL-6, interleukin-6; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; TNF, tumor necrosis factor.

bedding (Bed O'Cobs, Anderson Cob Division, Maumee, OH). Mouse Diet 9F 5020 (LabDiet, Madison, WI) and acidified tap water were provided *ad libitum*. Mice were inspected daily. All experimental protocols were approved by the Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health.

Full-length congenic B6.BR(*D1Mit5-D1Mit17*) (B6.BR-Ch1) mice were generated as described previously (21). Full-length congenic B6.BR(*D17Mit164-D17Mit2*) (B6.BR-Ch17) mice were generated as follows: B6 and BR mice were mated to generate B6BRF$_1$ animals and the F$_1$ males were backcrossed to B6 females. Mice carrying an ~77 Mb region of chromosome 17 from the BR strain, from *D17Mit164* at 3.92 Mb through *D17Mit2* at 80.98 Mb, were selected and used for subsequent backcrossing. Markers *D17Mit10*, *D17Mit23* and *D17Mit27* within this region were also genotyped to ensure that the entire segment would be preserved. Repeated backcrossing and selection was continued for each subsequent generation until N$_{10}$. Heterozygous generation 10 congenic animals were then intercrossed to yield animals homozygous for the BR chromosome 17 region on a B6 background.

Animals recombinant within the chromosome 17 congenic region were generated as follows: B6.BR-Ch17 males were crossed to B6 females to produce heterozygous F$_1$ animals; F$_1$ males were backcrossed to B6 females and the male progeny were genotyped to identify new recombinations. Males with the desired recombinations were bred to B6 females, and heterozygous progeny were intercrossed to yield homozygous recombinant lines.

Liver tumor induction and analysis

Heterozygous chromosome 17 recombinant mice were initially bred by crossing heterozygous recombinant males to B6 females, and progeny were genotyped to identify those mice with one copy of the entire desired region and those that were B6 at all genotyping markers. For each line, two of these B6 genotype males and females were kept as B6 sibling control mice. All mice with the entire desired recombinant region were also kept. This breeding procedure was followed until the recombinant line was bred to homozygosity and then subsequent mice were generated by crossing homozygous recombinant males to B6 females and genotyping progeny to ensure that all were heterozygous for the desired recombinant region.

Liver tumors were induced by a single intraperitoneal injection of DEN (Sigma, St Louis, MO; 0.1 μmol/g body wt) dissolved in trioctanoin (Sigma; 0.01 ml/g body wt) at 12 ± 1 days of age. As male mice are more susceptible than females to both spontaneous (5,6) and chemically induced (7,8) liver tumors, regardless of strain, males were killed at 32 weeks of age and females at 50 weeks by CO$_2$ asphyxiation. Livers were removed and weighed and all tumors >1 mm in diameter and visible on the surface of the liver were counted. Mean tumor multiplicities of full-length congenics were compared with inbred B6 mice, and chromosome 17 recombinants were compared with B6 sibling mice using the Wilcoxon rank sum test (22). For the congenic analysis, portions of liver were sampled at random for histology and for the recombinant analysis, liver tumors were randomly sampled. The selected tissues were fixed in buffered formalin, embedded and sections were stained with hematoxylin and eosin. Spleens were removed as a source of DNA, frozen on dry ice and stored at −80°C.

Genotyping

DNA was isolated from ~1 mm toe or tail clippings or ~1 mm^3 spleen. Tissue was digested in 500 μl lysis solution (1% sodium dodecyl sulfate, 150 mM NaCl, 100 mM ethylenediaminetetraacetic acid and 20 mM Tris–HCl, pH 8.0) and 15 μl (for toe or tail) or 25 μl (for spleen) proteinase K (Roche, Indianapolis, IN; 10 mg/ml, 0.3 mg/ml or 0.5 mg/ml, respectively, final) and incubated at 37°C overnight. Cellular debris was precipitated using 200 μl 6.25 M ammonium acetate and pelleted. DNA was precipitated with 700 μl isopropanol and washed with 500 μl 70% ethanol. The pellet was resuspended in 100 μl (for toe or tail) or 250 μl (for spleen) sterile water.

Microsatellite markers were amplified using 1 μl or 2 μl DNA (~100 ng), 184 nM each primer, 46 μM dNTPs, 10× polymerase chain reaction (PCR) buffer (Roche; 10 mM Tris–HCl, pH 8.3, 1.5 mM MgCl$_2$, 50 mM KCl) and 0.5 U *Taq* polymerase (Roche or enzyme purified in our lab by ammonium sulfate precipitation) in a final reaction volume of 21.7 μl. Reactions were incubated in an MJ PTC-200 thermal cycler (Bio-Rad, Hercules, CA) at 94°C for 3 min and then for 40 cycles of 94°C for 30 sec, 55°C for 40 sec, 72°C for 60 sec and 72°C for 5 min. The products were separated by electrophoresis through a 7% polyacrylamide gel. To generate B6.BR-Ch17 congenics, backcross progeny were genotyped at the following microsatellite markers: *D17Mit2; -10; -23; -27* and *-164*. Markers used to identify new recombinants along the chromosome 17 congenic region are listed in supplementary Table 1 (available at *Carcinogenesis* Online).

Recombinant breakpoint determination

Additional genotyping was performed in several recombinant lines to more precisely determine the location of breakpoints. Genotyping initially utilized

additional polymorphic microsatellite markers, following the procedure described above, and sequencing of known polymorphic SNPs identified from the Mouse Phenome Database (www.jax.org/phenome). These results were further supplemented by sequencing of non-coding portions of genes that were determined to be polymorphic in the minimal susceptibility region. Primers used for breakpoint sequencing are found in supplementary Table 2 (available at *Carcinogenesis* Online).

SNP and sequencing analysis

SNPs from B6, BR and C3H along the entire minimal susceptibility region were compiled from the Mouse Phenome Database. In addition, PCR primers were designed to amplify 500–700 bp regions of the 3′-untranslated region of chromosome 17 genes spanning the minimal susceptibility region. In some genes, the length of the 3′-untranslated region was inadequate. Consequently, 500–700 bp segments of the most 3′-intron of sufficient length were amplified. Supplementary Table 3 (available at *Carcinogenesis* Online) lists all non-coding primers that were used in this sequence analysis. DNA from B6, BR and C3H strains was initially amplified either exactly as for genotyping or with FailSafe PCR 2× PreMixes (Epicentre, Madison, WI) in place of Roche 10× PCR buffer and dNTPs, and a portion of the reaction was analyzed by electrophoresis in a 1% agarose gel. Successful reactions were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) using its standard protocol, except the final elution was performed twice with 20 μl sterile water. Approximately 35–50 ng of purified DNA was used in separate sequencing reactions for forward and reverse primers with 90 nM primer, Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA) and 5× buffer (400 mM Tris pH 9.0 and 10 mM MgCl$_2$, Applied Biosystems) in a final volume of 11 μl. Reactions were precipitated using 80 μl of 75% isopropanol, resuspended in 20 μl sterile water and electrophoresed in a 3730xl automated DNA sequencing instrument (Applied Biosystems) using 50 cm capillary arrays and POP-7 polymer. Sequences were analyzed and assembled with Phred/Phrap software (21,23). All reactions were performed in duplicate to confirm sequence differences.

Results

Generation and susceptibility of BR full-length congenics

The extremely high susceptibility of BR females and moderate susceptibility of BR males to DEN-induced hepatocarcinogenesis have been shown by linkage analysis to be primarily due to susceptibility loci on chromosomes 1 and 17 (19). To verify the presence of these loci and investigate any potential interaction between them, two separate full-length congenic lines were bred. Large portions of either chromosome 1 or 17 from the susceptible BR strain were moved onto a resistant B6 background. The generation of the B6.BR-Ch1 congenic has been described previously (20). To generate the full-length congenic for chromosome 17, B6BRF$_1$ males were backcrossed to B6 females for nine generations to yield N$_{10}$ progeny. With each generation, males were genotyped at five microsatellite markers spanning ~77 Mb of chromosome 17 from *D17Mit164* at 3.92 Mb to *D17Mit2* at 80.98 Mb, and males heterozygous for this entire region were bred to produce the next generation.

To assess the susceptibility of the full-length congenics to DEN-induced hepatocarcinogenesis, resistant B6, susceptible BR, homozygous congenic, heterozygous congenic and doubly heterozygous congenic mice were evaluated (Table I). The highly susceptible BR females had an average of 92 liver tumors, 13-fold more than the average of seven tumors seen in resistant B6 females ($P = 1 \times 10^{-10}$). Homozygous B6.BR-Ch1 females showed a 3-fold increase, whereas the B6.BR-Ch17 females had a 4-fold increase compared with the B6 females. The BR males had a moderate susceptibility with an average of 27 liver tumors, a 7-fold increase in tumor multiplicity compared with the average of four tumors in resistant B6 males ($P = 2 \times 10^{-7}$). B6.BR-Ch1 males showed a 7-fold increase, whereas B6.BR-Ch17 males demonstrated a 4.5-fold increase relative to B6 males. The increased susceptibility of both full-length BR congenics to DEN-induced liver tumors confirms the presence of susceptibility loci seen in this strain in the previous linkage analysis (19). Portions of liver were randomly chosen from each group and formalin fixed for histological analysis. In total, 155 tumors were evaluated. With the exception of one lymphoma, all tumors observed in liver sections were hepatocellular. Of these, 86% were adenomas or

Table I. Susceptibility of female and male BR full-length congenics[a]

Genotype	Female			Male		
	No. of mice	Mean liver tumors	P-value[b]	No. of mice	Mean liver tumors	P-value
B6	27	7 ± 7	—	37	4 ± 5	—
BR	33	92 ± 47	1×10^{-10}	36	27 ± 24	2×10^{-7}
B6.BR-Ch1	33	20 ± 21	0.004	30	28 ± 25	3×10^{-8}
B6 × B6.BR-Ch1	16	8 ± 8	0.4	16	12 ± 13	1×10^{-3}
B6.BR-Ch17	32	26 ± 18	2×10^{-6}	35	18 ± 15	8×10^{-8}
B6 × B6.BR-Ch17	33	26 ± 22	8×10^{-6}	39	18 ± 15	2×10^{-6}
B6.BR-Ch1×B6.BR-Ch17	33	54 ± 29	5×10^{-10}	39	31 ± 21	2×10^{-12}

[a]Mice received an intraperitoneal injection of DEN (0.1 μmol/g body wt) at 12 ± 1 days of age. Females were killed at 50 weeks of age and males at 32 weeks of age and tumors >1 mm in diameter on the surface of the liver were counted.
[b]Wilcoxon rank sum test was used to determine the P-value of each group compared with resistant inbred B6 mice. P-values are not adjusted for multiple comparisons.

a mixture of adenoma and carcinoma, the rest were carcinomas. The proportions of the subtypes did not depend on sex or genotype. A small number of tumors in B6 and the BR congenics had vascular invasion. Frequent fatty metamorphosis was seen in both normal and tumor tissue of B6.BR-Ch1 × B6.BR-Ch17 double heterozygotes.

The intermediate phenotype repeatedly seen in the B6 × B6.BR-Ch1 mice heterozygous for the chromosome 1 region (Table I and A.Bilger and N.R.Drinkwater, unpublished data) suggests that this susceptibility locus acts semidominantly in males and females. The high susceptibility seen in B6 × B6.BR-Ch17 mice heterozygous for the chromosome 17 region suggests that this locus acts semidominantly or dominantly (Table I). The doubly heterozygous B6.BR-Ch1 × B6.BR-Ch17 females, with one copy of each congenic region, demonstrated a 9-fold increase in liver tumors compared with resistant B6 females, whereas the B6.BR-Ch1 × B6.BR-Ch17 males showed an 8-fold increase compared with B6 males. These results indicate that the two susceptibility loci act additively in both sexes. The high susceptibility of these double heterozygotes reinforces the conclusion that these two susceptibility loci are responsible for a majority of the increase in susceptibility seen in the BR strain compared with B6. The effect of the chromosome 1 region was more significant than the effect of the chromosome 17 region in males, while the chromosome 17 region had a greater effect on hepatocarcinogenesis in females.

Generation and susceptibility of BR chromosome 17 recombinants
To begin to map more precisely the location of the chromosome 17 susceptibility locus, homozygous full-length B6.BR-Ch17 congenic males were crossed to B6 females and the resulting heterozygous F₁ males were backcrossed to B6. Male progeny were genotyped at nine microsatellite markers along the entire congenic region to identify new recombinants. A series of 16 ordered recombinant males were obtained that were heterozygous for new recombinations all along chromosome 17 (Figure 1).

To assess the susceptibility of this set of recombinants to liver tumors, inbred B6, inbred BR, B6 × B6.BR-Ch17 and heterozygous recombinants were generated and treated with DEN. During the course of breeding the heterozygous recombinants, mice that were B6 at all genotyping markers were also generated. Two of these mice of each gender from each recombinant line were kept to serve as the B6 sibling control group. All groups were compared with these B6 siblings. There was no difference in the susceptibility of these B6 sibling mice and the inbred B6 mice.

In females, one copy of the full-length chromosome 17 congenic region resulted in an average of 26 liver tumors per animal (Table II), a 4-fold increase compared with the average of six tumors seen in resistant B6 sibling females ($P = 3 \times 10^{-5}$). Susceptible heterozygous recombinant lines demonstrated between 16 and 45 liver tumors on average or a 3- to 7.5-fold effect compared with B6 siblings, completely recapitulating the increase seen in the full-length congenic. In males, one copy of the full-length chromosome 17 congenic region caused 31 liver tumors on average, a more moderate 2-fold
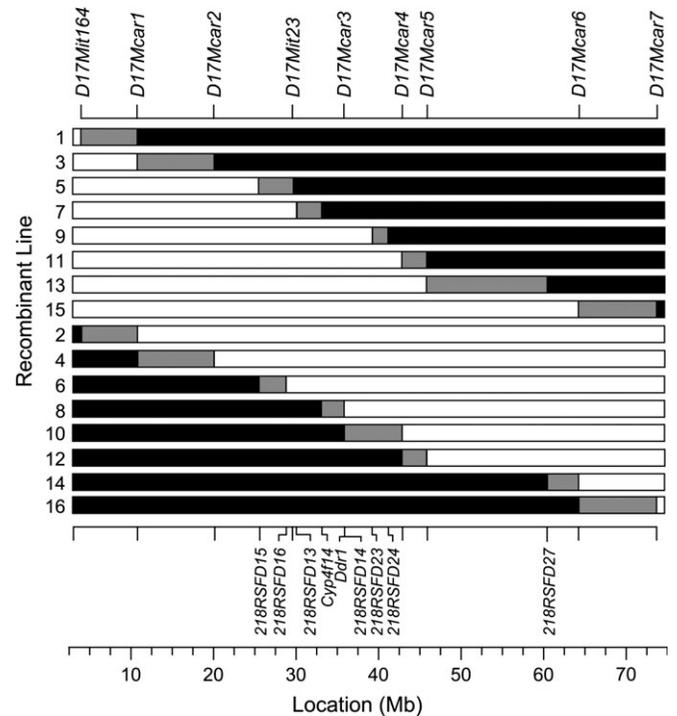

Fig. 1. Ordered series of 16 recombinant lines for chromosome 17. Sixteen ordered recombinant lines were bred, each containing a segment of chromosome 17 from the susceptible BR strain on a resistant B6 background. Microsatellite markers used for genotyping and their positions are indicated at the top. Additional sequencing markers used to further refine breakpoint locations are indicated at the bottom. Black regions indicate an area inherited from the B6 strain, white regions indicate a portion inherited from the BR strain and gray regions are areas of unknown genotype where recombinations have occurred.

increase in liver tumors compared with the average of 17 tumors in B6 sibling males. This 2-fold increase in the full-length chromosome 17 congenic males is slightly less than the 4.5-fold increase in liver tumors observed when these heterozygotes were first evaluated due to the elevated susceptibility of the B6 sibling control males. Males from the recombinant lines that exhibited susceptibility in females showed between 25 and 39 liver tumors on average, a 1.5- to 2-fold increase in liver tumor multiplicity compared with B6 siblings, also wholly recapitulating the effect of the full-length congenic region.

In the set of 16 recombinant groups, lines 1, 3, 5, 7, 8, 10, 12, 14 and 16 showed no difference in liver tumor susceptibility compared with B6 sibling mice in either females or males. All other recombinant lines were significantly more susceptible in females, even after P-values were adjusted for multiple comparisons ($P < 0.015$). P-values

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Table II. Susceptibility of female and male heterozygous chromosome 17 recombinants[a]

Genotype/line[b]	Females			Males		
	No. of mice	Mean liver tumors	*P*-value[c]	No. of mice	Mean liver tumors	*P*-value
B6 sibling[d]	32	6 ± 7	—	30	17 ± 21	—
B6	23	3 ± 4	0.1	34	16 ± 24	0.6
BR	24	162 ± 54	2×10^{-10}	21	56 ± 37	1×10^{-4}
B6 × B6.BR-Ch17	16	26 ± 19	3×10^{-5}	26	31 ± 23	0.005
1	31	7 ± 6	0.4	27	13 ± 19	0.4
3	14	4 ± 3	0.8	26	15 ± 13	0.8
5	12	8 ± 12	0.8	17	25 ± 24	0.3
7	19	12 ± 13	0.04	23	15 ± 23	0.6
9	21	33 ± 26	8×10^{-7}	25	25 ± 17	0.009
11	21	30 ± 27	2×10^{-5}	25	25 ± 26	0.05
13	25	27 ± 23	4×10^{-5}	27	35 ± 41	0.006
15	18	16 ± 14	0.001	24	29 ± 27	0.03
2	16	45 ± 32	1×10^{-6}	21	39 ± 29	0.004
4	15	26 ± 25	1×10^{-4}	20	34 ± 28	0.009
6	17	30 ± 28	9×10^{-6}	17	29 ± 24	0.08
8	24	9 ± 13	0.3	29	15 ± 15	0.9
10	26	9 ± 25	0.3	24	13 ± 11	1.0
12	12	3 ± 6	0.02	11	12 ± 21	0.2
14	25	9 ± 11	0.3	27	14 ± 16	0.8
16	14	4 ± 10	0.07	23	17 ± 20	0.7

[a]Mice were treated with an intraperitoneal injection of DEN at 12 ± 1 days of age. Females were killed at 50 weeks of age and males at 32 weeks of age. Tumors >1 mm in diameter on the surface of the liver were counted.
[b]All mice were heterozygous for the indicated recombinant region.
[c]Wilcoxon rank sum test was used to calculate the *P*-value of each group compared with B6 sibling mice. *P*-values are not adjusted for multiple comparisons.
[d]B6 sibling mice were B6 at all markers used for genotyping. Two such males and females from each recombinant line were kept for a B6 sibling control group.

for males from these same recombinant lines were marginally significant when adjusted for multiple comparisons, suggesting that the same region of chromosome 17 that caused the 3- to 7.5-fold increase in females also caused an increase in liver tumor multiplicity in males. Tumors were randomly chosen from each group and formalin fixed for histological analysis. In total, 77 tumors were analyzed, and ~75% were hepatocellular adenomas and the remaining 25% were mixed adenomas and carcinomas, independent of either strain or sex.

Additional genotyping was performed to further restrict the breakpoint regions in several recombinant lines and this information is included in Figure 1. This genotyping initially utilized polymorphic microsatellite markers. Further genotyping analyzed known polymorphic SNPs and non-coding regions of genes determined to be polymorphic during the haplotype analysis of the minimal susceptibility region. The ends of the minimal susceptibility region are set by the breakpoints in resistant lines 7 and 8, which have the largest portion of chromosome 17 from the BR strain that does not affect liver tumor susceptibility. The breakpoints in these lines were determined as precisely as possible and further supported by refinement of the breakpoints in susceptible lines 6 and 9, which have the smallest portion of chromosome 17 from the BR strain that causes an increase in liver tumor susceptibility. This additional mapping led to the conclusion that the chromosome 17 susceptibility locus must be between *218RSFD13* and *218RSFD14* or between 30.05 and 35.83 Mb. This single region accounts for the entire increase in susceptibility to liver tumors seen in the full-length chromosome 17 congenic compared with resistant B6 sibling controls.

Haplotype analysis of minimal susceptibility region
All of the SNPs with known alleles in the B6, C3H and BR strains in the minimal susceptibility region identified by the recombinant lines were assembled from the Mouse Phenome Database. The C3H strain was included because this strain does not have any loci that confer liver tumor susceptibility relative to B6 on chromosome 17. Thus, for the minimal susceptibility region, C3H is in essence an additional resistant strain. If the *Hcf1* susceptibility alleles were the result of an ancestral mutation, then it would be more likely to be found in regions where the BR strain has inherited an ancestral haplotype that

is different from both the B6 and C3H strains. The proximal two-thirds of the minimal region is virtually non-polymorphic in the BR and B6 strains (Figure 2A), while the BR and C3H strains are known to have the same *H2* haplotype, which is located in the distal portion of the minimal region from ~34.08 to 37.62 Mb. In addition, we assessed sequence variation specifically in non-coding portions of genes along the minimal susceptibility region among B6, C3H and BR mice. One hundred thirty-two segments of 500–700 bp from 3′-untranslated regions or introns of 120 genes throughout this region were sequenced in the resistant B6 and susceptible BR strains (supplementary Table 3 is available at *Carcinogenesis* Online). Any regions that were polymorphic in these two strains were also sequenced in the C3H strain. These sequence comparison studies determined that the proximal two-thirds of the minimal region were virtually identical in the B6 and BR strains (Figure 2B). In the remaining portion of the region, the B6 and BR strains were highly polymorphic. However, the C3H strain was virtually identical to the BR strain in this interval.

Only a very small number of loci were unique in the BR strain, the only strain containing a susceptibility locus on chromosome 17. Two of these unique regions are located at ~30.05 and 35.74 Mb in both comparisons (Figure 2). The non-coding region sequencing also identified two additional unique regions at ~33.04 and 34.50 Mb (Figure 2B). If the *Hcf1* mutation is the result of an ancestral mutation that the BR strain inherited during its generation, then it would probably be found in these small unique regions. However, the overwhelming majority of sequence is not unique to the distinctively sensitive BR strain in the minimal susceptibility region. This observation strongly suggests that the susceptibility locus is not the result of an ancestral mutation, but is most probably the result of a novel mutation that arose during generation of the BR strain. Consequently, all portions of the current minimal susceptibility region have an equal likelihood of containing the *Hcf1* mutation.

Discussion

The susceptibility of BR mice to hepatocarcinogenesis is primarily caused by two susceptibility loci, *Hcf1* and *Hcf2*, on chromosomes 17 and 1, respectively. These loci were initially identified through
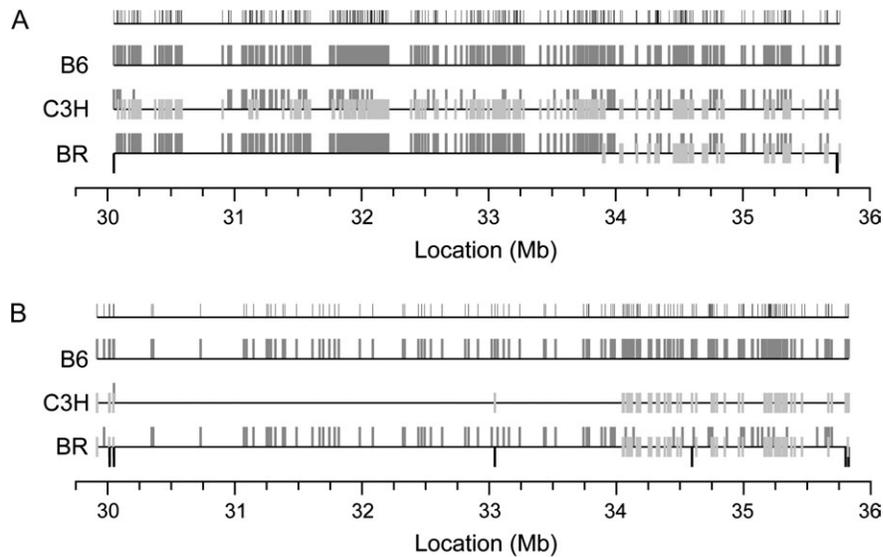
Fig. 2. Haplotype analysis of chromosome 17 minimal susceptibility region. The top line in each panel indicates the positions of the SNPs. For each strain, SNP alleles are indicated by both the shading of the vertical tic and its position with respect to the horizontal line. Sequences identical to those in B6 are indicated in dark gray (above the horizontal line), sequences different from B6 but identical in C3H and BR are in light gray (across the line) and sequences unique to BR are in black (below the line). Closely spaced SNPs may not be individually distinguishable in the representations for each strain. (**A**) SNPs with alleles in B6, C3H and BR were compiled from the Mouse Phenome Database. The data show two areas unique to BR at ∼30.05 and 35.74 Mb. (**B**) Segments of 3′-untranslated regions or the most 3′-introns of genes were sequenced. These data identify two additional areas unique to BR at ∼33.04 and 34.50 Mb.

a linkage analysis of crosses between the susceptible BR and resistant B6 strains and their existence has now been further validated through the analysis of congenic mice. Homozygosity for the full-length BR chromosome 1 region caused a 3-fold increase in liver tumor multiplicity in females and a 7-fold increase in males. The chromosome 17 region had a 4-fold effect in females and a 2- to 4.5-fold effect in males. Both of these regions appear to act at least semidominantly in both sexes. The high susceptibility seen in double heterozygotes, with one copy of each region, indicates that these two loci are responsible for the majority of the susceptibility in the BR strain. For the *Hcf1* locus on chromosome 17, a set of 16 recombinant lines covering the entire congenic region was generated, each containing a portion of chromosome 17 from the BR strain on a B6 background. Susceptible heterozygous chromosome 17 recombinants demonstrated between a 3- and 7.5-fold effect in females and between a 1.5- and 2-fold effect in males, completely recapitulating the increase seen in heterozygous full-length congenics. The analysis of these recombinants indicates that *Hcf1* is located in a single region from 30.05 to 35.83 Mb that is responsible for the increase in liver tumor sensitivity.

BR females are up to 50-fold more susceptible than females of all other inbred strains that have been evaluated (8). This effect is partly due to the loss of protection against liver tumor development typically offered by ovarian hormones: ovariectomized and intact BR females do not show a significant difference in liver tumor multiplicity (11). However, the fact that the two susceptibility loci are the major cause of susceptibility in BR females and males suggests that the same underlying pathway must be at work in both sexes. Work from other labs indicates that the protective effect of the ovaries is most probably mediated by estrogen. Chronic administration of estrogen has been shown to protect intact males (24) and ovariectomized females (25). Furthermore, progesterone was seen to have no effect on liver tumor incidence. As estrogen levels in females during diestrus have been shown to be only approximately twice that measured in males (26), estrogen could be exerting a protective effect in both sexes that is overwhelmed in males by the promotion of liver tumors caused by androgens (7,10,11,18,27). The increase in liver tumor multiplicity in BR males and females due to the chromosome 17 locus could therefore be caused by the loss of the same protective effect in both sexes. There is no difference in the levels of estrogen in B6 and BR females (M.H.Feld and N.D., unpublished data). The amounts and affinities of

estrogen receptor in the livers of B6 and BR mice are similar as well (13), suggesting that the chromosome 17 locus acts downstream of estrogen binding.

Estrogen may have either a direct or an indirect effect on hepatocarcinogenesis. When chimeras between the BR and B6 strains were treated with DEN, a majority of the tumors that developed originated from the BR strain (12). This result indicates that the BR susceptibility genes collectively have a cell-autonomous effect. The chromosome 17 locus may act downstream of estrogen binding and so *Hcf1* could cause cell-autonomous effects in the liver in response to estrogen. However, it is unknown whether only one locus or both *Hcf* loci act cell autonomously. Chimeras between C3H and B6 have similarly demonstrated the inherent susceptibility of the C3H strain to both spontaneous (28) and DEN-induced (29) liver tumors. As the C3H and BR strains appear to share a susceptibility locus on chromosome 1, it is possible that the chromosome 1 locus acts cell autonomously in both of these strains, whereas the chromosome 17 locus acts non-cell autonomously.

There is explicit evidence that estrogen may have an indirect effect on hepatocarcinogenesis: subcutaneous implantation of estrogen pellets decreases liver tumor incidence, whereas implantation of estrogen pellets in the spleen, which drains directly into the liver, does not (25). The change in expression of interleukin-6 (IL-6) in response to DEN is greater in males and ovariectomized females than in intact females (30), and the differences among these groups are reduced by the injection of estrogen. Knocking out IL-6 confers on males a resistance to hepatocarcinogenesis that is comparable with that of wild type and IL-6 knockout females. Naugler *et al.* (30) have hypothesized that the death of hepatocytes due to DEN causes Kupffer cells to release IL-6, which promotes compensatory hepatocyte regeneration, increasing the fixation of initiating mutations that result in liver tumors. Estrogen might protect livers from tumors indirectly by inhibiting the production of IL-6.

The multistage model of carcinogenesis, originally developed to describe tumor formation in the skin (31), can also be applied to tumor formation in the liver (14). This model divides tumor development into the three stages of initiation, promotion and progression. Ovarian hormones exert much of their protection during the promotion phase. Estrogen could act during the conversion stage of promotion (31), when an initiated cell divides and begins to express its preneoplastic

phenotype, by inhibiting production of IL-6 (30). Estrogen may also act later during the propagation stage of promotion, by inhibiting the proliferation or enhancing apoptosis of preneoplastic cells. Preneoplastic lesions in the liver have similar growth rates in B6 and C3H females, but grow more rapidly in BR females (11,15). These lesions grow more slowly in B6 and C3H females than in the corresponding males, but the growth rates of lesions in BR males and BR females are similar. Moreover, ovariectomy performed weeks after carcinogen treatment increases the growth rate of preneoplastic lesions in B6, C3H and B6C3F$_1$ females, but not in BR females (7,11,16–18). Further evidence that the modifiers act after initiation is provided by the observation that BR males haves a high spontaneous incidence of liver tumors (32) and that both sexes are susceptible to liver tumors induced by the direct-acting carcinogen *N*-ethyl-*N*-nitrosourea (8).

The liver tumor susceptibility locus *Hcf1* has so far been mapped to a region of chromosome 17 from 30.05 to 35.83 Mb, which corresponds to a section of human chromosome 6p21. Amplification of this portion of the p arm of chromosome 6 has repeatedly been found in human liver tumors. The proportion of tumors with this amplification varied from 20 to 61% and was found in patients with hepatitis B or C virus infection (33–36), as well as patients without hepatitis virus infection (37–39). An increase in the copy number of this region may indicate the presence of a dominantly acting oncogene. Additionally, this amplification was not associated with liver tumor grade, indicating that it could be an early event (40–42). Interestingly, amplification of chromosome arm 6p was also seen specifically in hepatic metastases from colorectal cancers (43,44), which are the leading cause of colorectal cancer deaths. Genetic aberrations in primary tumors and metastases from Dukes' stage C colorectal cancer patients with lymph node metastasis were compared with Dukes' stage D colorectal cancer patients with liver metastasis (44). Amplification of chromosome arm 6p was only significantly associated with Dukes' stage D and liver metastases. Consequently, identification of the causative mutation in the *Hcf1* locus could have wide-ranging effects on other diseases in addition to liver cancer.

The current minimal region contains ∼215 genes. Several genes in this region are potential candidates for *Hcf1*. *Cyp4F14* is a member of the cytochromes P450 4F subfamily. These enzymes are involved in arachidonic acid metabolism (45) and metabolize leukotriene B$_4$ into biologically less active metabolites (46). Leukotriene B$_4$ is a powerful promoter of inflammation, which can in turn lead to liver cancer (4). The sequence of *Cyp4F14* in mice has 95% sequence similarity with *Cyp4F1* in rats and both are expressed in the liver (45). The expression of this enzyme in rats is sex specific, with significantly higher expression in females (47). Moreover, its expression was shown to decrease after ovariectomy (47) and increase after exposure to aflatoxin B$_1$ (48), a known risk factor for liver cancer (4). *Cyp4F1* was also the first P450 found to be constitutively overexpressed in rat hepatomas (49).

Another candidate for *Hcf1*, *H2-Ke6*, is a member of the nicotinamide adenine dinucleotide-dependent 17β-hydroxysteroid dehydrogenase family of enzymes. It is expressed in the ovaries, testes and liver, in addition to other tissues (50). This family of enzymes efficiently catalyzes the oxidation of estradiol, testosterone and dihydrotestosterone as well as the reduction of estrone to form estradiol (51). These enzymes carry out a key reaction in the synthesis and metabolism of sex hormones and regulate the last step required to form all androgens and estrogens in both gonadal and non-gonadal tissues (52). Expression of *H2-Ke6* has previously been linked to the development of cysts in the livers and kidneys of mice.

A final candidate is tumor necrosis factor (TNF)-α, a proinflammatory cytokine. TNF can trigger the acute phase response and a cascade of other cytokines, and it also has a crucial role in the balance of hepatocyte proliferation and death (53). The transcription factor nuclear factor-kappa B is at least partly responsible for cell proliferation in response to TNF, and the activation of nuclear factor-kappa B in liver regeneration is primarily due to IL-6 induction. As previously stated, the production of IL-6 by non-parenchymal cells has been hypothesized to cause the gender disparity in DEN-induced liver tumors (30). In addition, TNF and IL-6 also have inhibitory effects on each other (54). IL-6 can inhibit TNF expression and TNF can block IL-6 induction of type II acute phase response genes and activation of signal transducer and activator of transcription signaling (55).

All of the exons of *Cyp4F14*, *H2-Ke6* and *Tnfa* and their splice sites have been sequenced in BR, B6 and C3H strains to look for unique mutations in the susceptible BR strain (S.Peychal and N.Drinkwater, unpublished data). This sequencing did not identify any unique mutations in either the exons or splice sites of these three candidate genes. In addition, the hepatic expression levels of these genes at 10 weeks of age were compared using microarrays. In B6, BR and BR chromosome 17 recombinant females, treated with DEN at 12 days and ovariectomized or sham operated at 6 weeks of age, the expression levels of the three genes were not significantly different among the strains. However, as there could be a causative difference in the expression of these genes at another time, or in another tissue, that could result in susceptibility to liver tumors, these genes cannot be excluded as the *Hcf1* locus.

Men have a 2- to 5-fold higher risk of developing liver cancer than women (1). At least some of this difference is due to the different hormonal environments, but it is not currently known what contribution, if any, is due to other complicating risk factors such as hepatitis virus infection or aflatoxin B$_1$ exposure. Inbred mice offer a simpler system in which to study liver cancer and the effect of sex hormones. Work with these mice has shown the stimulatory effect of androgens as well as the protective effect of ovarian hormones (7,9–11). The BR females are highly susceptible to liver cancer due, in part, to their unique lack of ovarian hormones' protection (11). Therefore, they offer a singular model to explore the suppressive pathways at work. Elucidation of how these pathways are disrupted in this unique strain may shed light on how these pathways function in all other strains and may uncover information of relevance to women and liver cancer development in humans as a whole.

Supplementary material

Supplementary Tables 1–3 can be found at http://carcin.oxfordjournals.org/

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