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A Potent Modifier of Liver Cancer Risk on Distal Mouse Chromosome 1: Linkage Analysis and Characterization of Congenic Lines

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ABSTRACT

The C3H/HeJ (C3H) and CBA/J (CBA) mouse strains are classical mouse models of cancer susceptibility, exhibiting high risks for both spontaneous and chemically induced liver cancer. By analysis of backcrosses and intercrosses between C3H or CBA and resistant B6 mice, we have mapped a potent modifier of hepatocellular carcinoma development to distal chromosome 1, linked to the marker *D1Mit33* with combined LOD_W scores of ~5.9 (C3H) and 6.5 (CBA). We previously identified this region as one of two that modify susceptibility in the more distantly related C57BR/cdJ (BR) strain. Congenic B6.C3H(*D1Mit5-D1Mit17*) and B6.BR(*D1Mit5-D1Mit17*) mice developed significantly more liver tumors than B6 mice did (6- to 13-fold, $P < 10^{-11}$, in males; 3- to 4-fold, $P < 10^{-3}$, in females). Thus, distal chromosome 1 carries one or more genes that are sufficient to confer susceptibility to liver cancer.

THE C3H/HeJ (C3H) and CBA/J (CBA) mouse strains are classical models of liver carcinogenesis, highly susceptible to both spontaneous and carcinogen-induced liver cancer (Andervont 1950; Flaks 1968; Smith *et al.* 1973). The genetic basis for their susceptibility has not been established. Identifying the number and types of genes underlying their susceptibility is likely to have public health implications, as liver cancer in C3H-derived $B6C3F_1$ mice is the single most common carcinogenic response to the >500 compounds that have been tested in chronic bioassays by the National Toxicology Program (Ashby and Tennant 1991; http://ntp-server.niehs.nih.gov/; January 2004).

By 2 years of age, 30–50% of C3H mice spontaneously develop hepatocellular carcinoma (HCC), the most common form of liver cancer in mice and humans (Storer 1966; Smith and Walford 1978). In contrast, <5% of 2-year-old B6 mice develop HCC (Frith and Wiley 1982). On the basis of modeling studies, we have suggested that the majority (~85%) of the difference in susceptibility between B6 and C3H is controlled by one locus (Drinkwater and Ginsler 1986). This quantitative trait locus (QTL) has been named *Hcs7* (*Hepatocarcinogen sensitivity 7*) on the basis of a preliminary report of its location (Bennett *et al.* 1993).

Hcs7 appears to control early stages of tumor development. Preneoplastic lesions, induced by treatment of male mice with *N,N*-diethylnitrosamine (DEN) or *N*-ethyl-*N*-nitrosourea (ENU) at 12–15 days of age, grow about twofold faster in 12- to 32-week-old C3H mice than in B6 mice (Hanigan *et al.* 1988; Pugh and Goldfarb 1992). The implication that the net growth of preneoplastic lesions in intact B6 livers is limited is supported by the results of partial hepatectomy of DEN-treated livers. The growth stimulus provided by partial removal of the liver causes a dramatic increase in lesion number and size in B6, but not C3H, mice (Hanigan *et al.* 1990), suggesting that partial hepatectomy activates in B6 a growth pathway that is already active in C3H lesions. This strain-specific difference in growth control is also evident in untreated livers that have undergone partial hepatectomy. The level of DNA synthesis in these normal hepatocytes is over twofold higher in C3H mice than in B6 mice (Bennett *et al.* 1995), indicating that *Hcs7* may play a role in normal hepatocyte growth control.

Hcs7 does not significantly affect carcinogen metabolism and tumor initiation. C3H mice are more susceptible than B6 to liver tumor induction by a variety of carcinogens that differ in their metabolic activation, and they are also more susceptible to spontaneous tumors (Nagasaki *et al.* 1975; Drinkwater and Ginsler 1986; Hoover *et al.* 1986; Wiseman *et al.* 1986). In addition,

This article is dedicated to the memory of our late colleague, Kristin M. Liss.

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similar numbers of DNA adducts and preneoplastic lesions form in the livers of carcinogen-treated B6 and C3H mice (Drinkwater and Ginsler 1986). Although 12-week-old C3H mice develop 25-fold more detectable preneoplastic foci than do age-matched B6 mice, this difference diminishes dramatically with age. This result suggests that similar numbers of lesions are initiated in B6 and C3H livers, but that B6 hepatic foci take longer on average to grow to detectable size, supporting the hypothesis that *Hcs7* controls preneoplastic growth (Hanigan *et al.* 1988).

Studies of chimeric mice indicate that *Hcs7* affects tumorigenesis from within the liver, probably at the level of the hepatocyte (Condamine *et al.* 1971; Lee *et al.* 1991). In chimeric mice derived from aggregated C3H and B6 embryos, spontaneous and DEN-induced tumors develop mainly from C3H hepatocytes—even in livers derived predominantly from B6 hepatocytes (Condamine *et al.* 1971; Lee *et al.* 1991). In addition, the effect of *Hcs7* appears tissue specific. C3H mice are more susceptible than B6 to tumorigenesis in the liver, but they are more resistant than B6 to colon cancer and comparable to B6 in susceptibility to lung cancer (Turusov *et al.* 1982; Malkinson and Beer 1983; Drinkwater and Ginsler 1986).

C57BR/cdJ (BR) mice, originally derived from the same breeding pair that generated B6 mice (Beck *et al.* 2000), are up to 50-fold more susceptible than B6 to liver tumorigenesis (Kemp and Drinkwater 1989). The loci responsible for this difference have been mapped to chromosome 17 (*Hcf1*) and distal chromosome 1 (*Hcf2*; Poole and Drinkwater 1996). Analysis of chimeras showed that the net effect of these factors is intrinsic to the liver and may be cell autonomous (Chiaverotti and Drinkwater 2003). A number of other loci, on chromosomes 2 (*Hcs4*), 4 (*Hcr1*), 5 (*Hcs5*), 7 (*Hcs1*), 8 (*Hcs2*), 10 (*Hcr2*), 12 (*Hcs3*), and 19 (*Hcs6*) have been implicated in the control of liver tumorigenesis on the basis of mapping crosses involving a variety of strains and carcinogens (Gariboldi *et al.* 1993; Manenti *et al.* 1994; Lee *et al.* 1995). Some of these loci may also control the growth rate of preneoplastic cells, which differs among the strains used in these crosses (A/J, DBA/2, BALB/c, and C3H; Dragani *et al.* 1987, 1991; Lee and Drinkwater 1995b).

We report here the first linkage analyses of a QTL that explains most of the difference in liver cancer susceptibility between B6 and C3H or CBA mice. We have mapped *Hcs7* to distal chromosome 1 by analysis of backcrosses and intercrosses between B6 mice and both susceptible strains. We have also generated congenic animals that carry, on a B6 background, a 70-cM region of distal chromosome 1 derived from either C3H or BR. These congenic mice confirm the location, potency, and independent action of the C3H chromosome 1 modifier.

MATERIALS AND METHODS

Mice: B6, BR, C3H, and CBA mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our facilities. All mice were housed in plastic cages on corncob bedding (Bed O'Cobs, Anderson Cob Division, Maumee, OH), fed Wayne Breeder Blox (Figure 1; Table 1; 11% fat; Continental Grain, Chicago) or Purina 5020 (Table 2; 9% fat; St. Louis) diet, and given acidified tap water *ad libitum*. Mice were inspected daily and weighed monthly.

Congenic B6.C3H(*D1Mit5-D1Mit17*) (abbreviated as B6.C3H-Ch1) and B6.BR(*D1Mit5-D1Mit17*) (B6.BR-Ch1) were generated as follows. B6 and C3H or B6 and BR mice were mated to yield $B6C3F_1$ or $B6BRF_1$ animals. F_1 males were then backcrossed to B6 females. Mice carrying an ~70-cM region of distal chromosome 1 derived from the C3H or BR strains were selected for additional backcrossing. The marker loci *D1Mit5* and *D1Mit17* were used to select the endpoints of the congenic region. Four to five independent lines were maintained during the generation of B6.C3H-Ch1 and B6.BR-Ch1 congenics. After two more rounds of mating, N_4 congenic male mice from each line were crossed to B6 females to generate experimental progeny (Table 1). N_4 congenic mice were also used in continued backcrossing to yield animals (N_{10}) that were then intercrossed to yield fully homozygous animals carrying the selected C3H or BR region on a B6 background (B6.C3H-Ch1 and B6.BR-Ch1; Table 2). One of each set of congenic lines was chosen for further analysis on the basis of phenotypic validation in the N_4 backcross, progeny testing, and thorough genotypic validation at N_{10}.

Tumor induction and assessment: Tumors were induced by a single intraperitoneal injection of DEN (Eastman Kodak, Rochester, NY; 0.05 μmol/g body weight for $B6CBF_1 \times B6$ backcross and $B6CBF_2$ intercross mice; 0.1 μmol/g body weight for all other mice except $B6C3F_2$; Drinkwater and Ginsler 1986) or ENU (0.25 μmol/g body weight for $B6C3F_2$ mice; Drinkwater and Ginsler 1986) dissolved in trioctanoin (Pfalz and Bauer, Stamford, CT or Sigma, St. Louis) 12 ± 1 days after birth. Male mice were killed by CO_2 asphyxiation at 31–32 weeks of age; females were killed at 49–50 weeks. Livers were removed and weighed; all tumors with diameters >2 mm ($B6C3F_1 \times B6$ backcross mice; Drinkwater and Ginsler 1986) or 1 mm (all other mice; Hanigan *et al.* 1990) on the surface of the liver were counted. Liver tumors were sampled at random and fixed in buffered formalin, and embedded sections were stained with hematoxylin and eosin. Spleens were collected as a source of DNA and frozen on dry ice. All tumors in a given cross were scored by a single observer blind to genotype.

Genotyping: Spleen DNA was prepared as follows: ~5 mm^3 of spleen was placed in 500 μl of a lysis solution (1% SDS, 150 mm NaCl, 100 mm EDTA, 20 mm Tris-Cl, pH 8.0) plus 25 μl proteinase K (10 mg/ml; 0.5 mg/ml final) and incubated at 55° for 3–20 hr. Cellular debris was precipitated with 250 μl 6.25 m ammonium acetate and pelleted. DNA was precipitated from the supernatant with 700 μl 100% isopropyl alcohol, and the DNA pellet was washed with 70% ethanol. The resulting genomic DNA pellet was resuspended in 250 μl of TE (10 mm Tris, 1 mm EDTA, pH 8.0).

Microsatellite markers (McAleer *et al.* 1992; Dietrich *et al.* 1996) were amplified using 1 or 2 μl of spleen DNA (~100 ng), 125–190 nm each primer, 50 μm dNTPs (Amersham, Piscataway, NJ), PCR buffer (Roche, Indianapolis; 10 mm Tris/HCl, 1.5 mm $MgCl_2$, 50 mm KCl, pH 8.3), and 0.024 units/μl Taq polymerase (Roche) in a total reaction volume of 20.5 (1 μl spleen DNA) or 21.5 (2 μl DNA). The reactions were incubated in thermocyclers at 94° for 3 min; followed by 40 cycles of 94° for 30 sec, 55° for 40 sec, and 72° for 60 sec; and

followed by 7 min at 72°. The products were separated by electrophoresis through a 7% acrylamide gel.

B6C3F$_1$ × B6 backcross progeny were genotyped at the following 107 markers: *D1Mit1*, *-5*, *-10*, *-13*, *-14*, *-15*, *-17*, *-26*, *-36*; *D2Mit1*, *-7*, *-13*, *-48*, *-49*, *-53*, *-74*; *D3Mit3*, *-7*, *-11*, *-17*, *-19*, *-42*, *-45*, *-55*; *D4Mit12*, *-13*, *-39*, *-42*; *D5Mit11*, *-24*, *-32*, *-63*; *D5Nds2*; *D6Mit1*, *-10*, *-14*, *-15*, *-16*, *-25*, *-29*; *D7Mit7*, *-21*, *-56*; *D7Nds1*, *-4*, *-5*; *D8Mit4*, *-8*, *-13*, *-16*, *-33*, *-42*, *-46*; *D9Mit2*, *-4*, *-12*, *-17*, *-18*, *-19*; *D9Nds2*; *D10Mit3*, *-10*, *-12*, *-14*, *-31*; *D10Nds1*; *D11Mit2*, *-5*, *-12*, *-14*, *-20*, *-41*; *D12Mit5*, *-34*, *-46*; *D12Nds2*, *-11*; *D13Mit3*, *-8*, *-13*, *-35*; *D14Mit5*, *-7*, *-14*, *-28*; *D15Mit6*, *-42*; *D15Nds2*; *D16Mit4*, *-9*, *-30*; *D17Mit1*, *-3*, *-6*, *-10*, *-18*, *-23*, *-35*; *D18Mit4*, *-8*, *-9*, *-17*, *-22*, *-33*; and *D19Mit1*, *-11*, *-16*. A subset of 34 animals, randomly chosen, was tested at every marker locus. The remaining 24 progeny were tested only at marker loci on chromosomes showing significant linkage to the tumor multiplicity phenotype. B6C3F$_2$ mice were typed at the following 50 markers: *D1Mit3*, *-5*, *-7*, *-13*, *-14*, *-15*, *-17*, *-21*, *-23*, *-26*, *-33*, *-34*, *-36*, *-46*, *-54*, *-61*; *D2Mit7*, *-13*, *-48*; *D3Mit7*, *-11*, *-17*; *D4Mit12*; *D5Nds2*; *D5Mit24*; *D6Mit33*, *-29*; *D7Nds1*; *D8Mit4*, *-33*; *D9Mit4*; *D10Mit68*; *D11Mit14*, *-20*, *-41*; *D12Mit5*, *-46*; *D13Mit3*, *-13*, *-35*, *-51*; *D14Mit14*, *-28*; *D15Mit2*, *-6*; *D16Mit4*; *D17Mit3*, *-23*, *-68*; and *D18Mit17*, *-33*.

Fifty-three B6CBF$_1$ × B6 backcross progeny were genotyped at the following 74 marker loci: *D1Mit3*, *-5*, *-13*, *-17*, *-30*, *-33*, *-60*, *-113*, *-150*; *D2Mit1*, *-35*, *-48*, *-49*, *-57*, *-62*, *-148*; *D3Mit62*, *-6*, *-9*, *-11*, *-14*, *-17*, *-19*; *D4Mit9*, *-16*, *-33*; *D5Mit61*, *-95*; *D5Nds2*; *D6Mit1*, *-9*, *-10*, *-15*, *-17*, *-25*; *D7Mit34*, *-56*; *D7Nds2*, *-4*; *D8Mit3*, *-41*, *-88*; *D9Mit2*, *-6*, *-10*; *D10Mit3*, *-10*, *-31*, *-72*; *D11Mit19*, *-23*; *D11Nds1*; *D12Mit5*, *-12*, *-20*, *-34*; *D13Mit3*, *-13*, *-30*; *D14Mit7*, *-14*, *-28*, *-62*; *D15Mit3*, *-43*; *D16Mit9*, *-30*; *D17Mit16*, *-38*, *-70*; *D18Mit4*; and *D19Mit10*, *-13*, *-31*. Ninety-five B6CBF$_2$ intercross progeny were genotyped at the following 12 marker loci: *D1Mit3*, *-5*, *-13*, *-17*, *-33*, *-60*, *113*, *-150*; *D12Mit5*, *-12*, *-34*; and *D13Mit13*.

Spleen DNA from 138 B6 × B6.C3H-Ch1 and 149 B6 × B6.BR-Ch1 N$_5$ progeny were genotyped at the following markers: *D1Mit5*; *D1Mit285* or *-89* (both at 63 cM); *D1Mit33*; and *D1Mit17* or *-117* (both at 106 cM).

Two or three animals from the B6.C3H-Ch1 and B6.BR-Ch1 homozygous congenic lines at generation N$_{10}$ were tested at marker loci spaced approximately every 5 cM throughout the congenic region. The markers used for B6.C3H-Ch1 were *D1Mit64*, *-66*, *-231*, *-211*, *-233*, *-5*, *-19*, *-23*, *-215*, *-83*, *-10*, *-135*, *-285*, *-91*, *-218*, *-100*, *-105*, *-33*, *-399*, *-15*, *-13*, *-206*, *-166*, *-461*, *-17*; markers used for B6. BR-Ch1 were *D1Mit5*, *-19*, *-23*, *-215*, *-83*, *-10*, *-135*, *-285*, *-105*, *-33*, *-399*, *-143*, *-206*, *-17*. Underlining indicates markers that were used to identify breeders during backcrossing to generate the congenic lines.

Linkage analysis: We used a nonparametric approach to assess linkage between the marker loci and the quantitative trait loci that determine liver tumor multiplicity (Kruglyak and Lander 1995; Poole and Drinkwater 1996). For backcross and intercross mice, the data for each marker were analyzed using the Wilcoxon rank sum or Jonckheere-Terpstra tests, respectively, to obtain the test statistic Z_W (Lehman 1998). The genome-wide, null distribution of $|Z_W|$ was determined empirically for each experiment by permutation of the phenotypic data (100,000 permutations for each cross) as described by Churchill and Doerge (1994). For each permutation, $max_{cross}(|Z_W|)$ was recorded and this distribution was used to determine the genome-wide significance (two-sided) for linkage to each marker (Lystig 2003). Linkage of markers to minor quantitative trait loci was assessed by a conditional permutation test in which the data were stratified by the genotype at the marker nearest the major quantitative trait locus (Doerge and Churchill 1996). This analysis should also reveal interactions between major and minor loci. Equivalent LOD (logarithm of odds) scores (Kruglyak and Lander 1995), LOD_W, were estimated from $LOD_W = 0.5\ (\log_{10} e)\ (Z_W)^2$. These analyses were performed using Qlink 3.2 software, which is available from the authors (http://mcardle.oncology.wisc.edu/qlink).

RESULTS

B6C3F$_1$ × B6 backcross and B6C3F$_2$ intercross: The high sensitivity of C3H mice is governed largely by a single locus (Drinkwater and Ginsler 1986). To map this QTL, we generated 58 B6C3F$_1$ × B6 backcross male progeny, injected them with DEN at 12 days of age, and counted their liver tumors at 32 weeks of age. The tumor multiplicity phenotypes were correlated with genotypes at 107 microsatellite markers spread at ~15-cM intervals throughout the 19 autosomes. (Previous analysis of F$_1$ mice had shown that the locus was not carried by the X or Y chromosomes; Drinkwater and Ginsler 1986.) Segregation analysis reveals a significantly linked region centered at *D1Mit15* at 88 cM on chromosome 1, with a LOD_W of 3.06 (genome-wide *P* value = 0.0067; Figure 1A). B6C3F$_1$ × B6 mice heterozygous for the C3H allele at *D1Mit15* developed twofold more tumors than their siblings that were homozygous B6 at this locus (40 ± 20 *vs.* 20 ± 21). Inbred C3H and B6 mice, treated in parallel, developed 78 ± 30 and 1.4 ± 1.6 tumors, respectively.

We independently tested this region's ability to modify liver tumor multiplicity and determined the effect of locus dosage by analyzing 57 B6C3F$_2$ intercross mice treated with ENU. Unlike DEN, which requires metabolic activation, ENU is a direct-acting alkylating agent. However, the two carcinogens yield identical patterns of ethylated bases in DNA (Beranek *et al.* 1980) and highly similar strain distribution patterns for liver tumor induction among BXH recombinant inbred strains (Lee and Drinkwater 1995a). Intercross mice were injected intraperitoneally at 12 days and killed at 32 weeks of age. Again, C3H alleles on distal chromosome 1 were most tightly linked with liver tumor susceptibility (Figure 1A). Specifically, *D1Mit13* at 63 cM yielded a significant LOD_W score of 2.85 (P_{genome} = 0.007; Figure 1A). This susceptibility locus appears semidominant. B6C3F$_2$ mice heterozygous for the C3H allele at *D1Mit13* developed threefold more tumors than B6 homozygotes did (10 ± 10 *vs.* 3.1 ± 5), and homozygosity for the C3H allele increased the number of tumors an additional twofold (19 ± 14 *vs.* 10 ± 10). Inbred C3H and B6 mice, treated in parallel with ENU, developed 20 ± 12 and 1.5 ± 1.6 tumors, respectively.

No other loci were found to interact significantly with the B6 or C3H alleles at *D1Mit13*. In this intercross analysis, mice lacking C3H alleles on distal chromosome 1 were generally not typed elsewhere. Therefore, the interaction of recessive B6 alleles on chromosome 1 with recessive C3H alleles elsewhere might not have

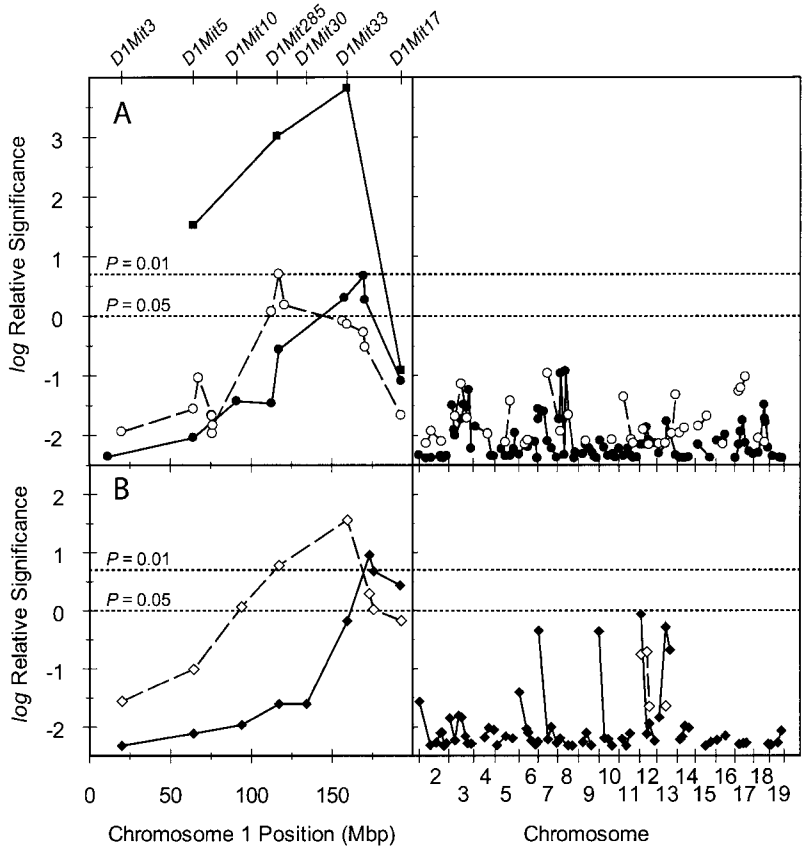

FIGURE 1.—A modifier of liver tumor multiplicity on distal chromosome 1. The log relative significance [log(0.05/genome-wide *P* value)] is plotted for markers on chromosome 1 (left) and for markers on all other chromosomes (right). Values corresponding to genome-wide *P* values of 0.05 and 0.01 are indicated by the dotted horizontal lines. (A) C3H crosses: solid circle, $B6C3F_1 \times B6$; open circle, $B6C3F_2$; solid square, B6.C3H-Ch1 N_5 backcross. (B) CBA crosses: solid diamond, $B6CBF_1 \times B6$; open diamond, $B6CBF_2$.

been detected. Interactions with dominant C3H alleles elsewhere should have been detected in the backcross.

$B6CBF_1 \times B6$ backcross and $B6CBF_2$ intercross: The CBA inbred strain was derived from the same C line as C3H and is almost identically susceptible to liver cancer, whether spontaneous or induced by a variety of carcinogens (GRASSO and HARDY 1975; DRINKWATER 1989). To map the CBA susceptibility loci, 53 $B6CBF_1 \times B6$ backcross and 95 $B6CBF_2$ intercross male progeny were injected with DEN at 12 days of age and tumors were counted at 32 weeks of age. The tumor multiplicity phenotypes of 53 backcross animals were correlated with their genotypes at 74 marker loci at ~20-cM intervals. Marker *D1Mit113* at 93 cM on chromosome 1 yielded a significant LOD_W score of 3.29 ($P_{genome} = 0.0036$; Figure 1B). The results of the F_2 intercross confirm the presence of a single strong modifier on chromosome 1. Significant linkage spanned the region between *D1Mit13* and *D1Mit17*, completely overlapping the susceptibility region in C3H, with a peak LOD_W score of 3.21 at *D1Mit33* at 82 cM ($P_{genome} = 0.0011$; Figure 1B). Animals carrying the dominant CBA allele developed between 2.5- and 3-fold more tumors than B6 homozygotes did (106 ± 43 *vs.* 42 ± 40, intercross; 69 ± 38 *vs.* 24 ± 29, backcross). Inbred CBA and B6 mice, treated in parallel, developed 166 ± 157 and 4 ± 6 tumors, respectively. No other loci were found to interact significantly with the chromosome 1 modifier.

Congenic backcross: The above mapping crosses between B6 and C3H or CBA and previous mapping crosses between B6 and BR (POOLE and DRINKWATER 1996) identified modifiers of liver cancer risk on distal chromosome 1. To verify the existence of these modifiers and test their ability to act alone, we generated congenic animals carrying C3H or BR chromosome 1 regions on a B6 background. We selected for C3H or BR alleles at four marker loci spanning a 70-cM region of chromosome 1, from *D1Mit5* to *D1Mit17*. After three generations of backcrossing, mice from four to five independent congenic lines (then at N_4) were crossed to B6 to generate N_5 backcross progeny. (On average, unlinked C3H or BR alleles outside the congenic region should compose only ~3% of the genome in these N_5 congenics, with each subline carrying a different complement of residual heterozygosity.) The N_5 congenics were treated with DEN and their tumors were counted, yielding additional mapping data and an initial assessment of the allele's ability to act independently. Among 138 mice, progeny heterozygous for C3H or BR chromosome 1 alleles developed 4- to 5-fold more tumors than their homozygous B6 siblings did (Table 1), suggesting that the chromosome 1 locus acts independently of other alleles in the donor strain.

Many of the backcross progeny carried newly recombinant chromosomes in the large congenic region. These novel recombinants were used to map the mod-

TABLE 1

Linkage of DNA markers to liver tumor susceptibility in congenic backcross mice

	Position[a]				Liver tumor multiplicity (N)[d]	
Marker	cM	Mbp	P_{genome}[b]	LOD_W[c]	B6/B6	B6/C3H or B6/BR
B6.C3H-Ch1 N_5						
D1Mit5	32.8	64.5	0.00114	2.78	6.7 ± 12 (56)	14.2 ± 17 (61)
D1Mit285	63.1	116.0	<0.0001	4.28	5.9 ± 12 (67)	14.5 ± 16 (67)
D1Mit33	81.6	159.0	<0.0001	5.08	4.4 ± 6 (68)	16.5 ± 18 (65)
D1Mit17	106	189.3		0.35	7.8 ± 12 (64)	12 ± 14 (60)
B6.BR-Ch1 N_5						
D1Mit5	32.8	64.5	0.027	1.53	1.4 ± 2.1 (59)	5.2 ± 9.7 (86)
D1Mit285	63.1	116.0	0.0106	1.90	1.4 ± 2.1 (63)	5.3 ± 9.8 (84)
D1Mit33	81.6	159.0	<0.0001	4.52	1.1 ± 1.9 (71)	5.9 ± 10 (77)
D1Mit17	106	189.3	0.0047	2.20	2.7 ± 8.6 (64)	4.3 ± 6.9 (84)

Male N_5 backcross mice were treated at 12 days of age with DEN (0.1 μmol/g body weight), and liver tumors were enumerated at 32 weeks of age.

[a] Positions of the markers on the genetic map (cM) were retrieved from the Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory (http://www.informatics.jax.org/; June, 2003). Marker positions on the physical map (Mbp, megabase pairs) were retrieved from the Mouse Ensembl Database (http://www.ensembl.org/Mus_musculus). The position of *D1Mit17* was estimated from radiation hybrid data on MGD.

[b] Genome-wide significance level.

[c] Threshold values ($P = 0.05$) for the B6.C3H-Ch1 and B6.BR-Ch1 backcrosses were 1.27 and 1.28, respectively.

[d] Values in the table for each marker genotype are the mean liver tumor multiplicity ±SD (number of mice).

ifier. Once again, peak linkage was near *D1Mit33*, with a LOD_W of 5.08 ($P < 10^{-4}$) for B6.C3H-Ch1 and a LOD_W of 4.52 ($P < 10^{-4}$) for B6.BR-Ch1. The data for all of the C3H and CBA mapping crosses are shown in Figure 1. Each cross yielded a highly significant LOD_W for distal chromosome 1. Combined, the three C3H crosses yield a LOD_W of 11.0, and the two CBA crosses yield a LOD_W of 6.5. Combining our previous linkage results for crosses between B6 and BR mice (Poole and Drinkwater 1996) with those for the B6.BR-Ch1 backcross yields a peak LOD_W score of 11.2 at *D1Mit33*.

Susceptibility of congenic mice: We continued backcrossing the congenic animals carrying C3H or BR chromosome 1 regions to B6 animals and assessed the heterozygous and homozygous effects of each congenic region in a single 10th-generation line (N_{10}; 0.1% unlinked donor genome). At this generation, the congenic region consisted of the selected 70-cM interval and up to 40 additional megabase pairs proximal to *D1Mit5*.

We found that the C3H and BR chromosome 1 regions impart dramatic susceptibility to both males and females (Table 2). Homozygosity for 70 cM of C3H chromosome 1 caused congenic males to develop 13-fold more tumors than B6 males ($P < 10^{-11}$) and congenic females to develop 4-fold more tumors than B6 females ($P < 10^{-7}$). [Similar results were obtained with B6, C3H, and B6.C3H-Ch1 animals fed a diet containing 6% rather than 9% fat (data not shown).] These increases account for most of the difference in susceptibility between B6 and C3H, for both genders. Specifically, the 13-fold effect in B6.C3H-Ch1 males accounts for 78% of the 27-fold effect between the B6 and C3H strains (in terms of relative risk), and the 4-fold effect in B6.C3H-Ch1 females accounts for 86% of the 5-fold effect between strains. Homozygosity for BR chromosome 1 resulted in a 6-fold increase in B6.BR-Ch1 males ($P < 10^{-8}$) and a 3-fold increase in B6.BR-Ch1 females relative to B6 ($P < 10^{-3}$). The effect in males accounts for 100% of the 6-fold difference in susceptibility between B6 and BR, but the 3-fold effect in females accounts for only 42% of the 14-fold difference between B6 and BR females. The discrepancy in females is due to susceptibility alleles on chromosome 17 (Poole and Drinkwater 1996). Chromosome 1 alleles from both C3H and BR appear semidominant in females and dominant in males.

Tumors induced in parental and congenic mice were selected randomly and assessed histopathologically. The tumors were all hepatocellular in origin, with the exception of one cholangioma and three sections that exhibited nodules consistent with lymphoma. Among the 211 hepatocellular tumors examined, approximately equal numbers were diagnosed as adenomas and carcinomas. The distribution between tumor types was independent of gender or strain. Hematoxylin- and eosin-stained liver sections from B6, B6.C3H-Ch1 congenic, and C3H mice were also scored for eosinophilic inclusions. Although commonly found in B6 hepatic lesions (Kakizoe *et al.* 1989), previous results suggested that these inclusions do not segregate with resistance to liver tumorigenesis (Lee and Drinkwater 1995a). We observed many inclusions in susceptible B6.C3H-Ch1 livers, confirming the

TABLE 2

Liver tumor susceptibility in inbred parental and chromosome 1 congenic mice

Strain	Liver tumor multiplicity (N)[a]	
	Male	Female
B6	4.4 ± 4.7 (37)	6.6 ± 6.8 (24)
C3H	119 ± 39[b,c] (34)	34 ± 26 (24)
B6.C3H-Ch1	54 ± 28[b,d] (32)	27 ± 24[e] (47)
B6 × B6.C3H-Ch1	60 ± 27[c] (34)	15 ± 16[e] (35)
BR	27 ± 24 (36)	93 ± 47[f] (32)
B6.BR-Ch1	28 ± 25[d] (30)	20 ± 21[f,g] (34)
B6 × B6.BR-Ch1	24 ± 29 (20)	7.9 ± 8.2[g] (16)

Mice were treated at 12 days of age with DEN (0.1 μmol/g body weight); males and females were killed at 32 and 50 weeks, respectively, for enumeration of liver tumors. Mean liver tumor multiplicities for all groups other than B6 × B6.BR-Ch1 female mice differed from those for sex-matched B6 mice ($P < 10^{-3}$, Wilcoxon rank sum test). Paired footnotes *b–g* indicate significant differences between the two groups by the Wilcoxon rank sum test.

[a] Values in the table are the mean liver tumor multiplicity ± SD (number of mice).

[b] $P < 10^{-5}$.

[c] $P < 10^{-4}$.

[d] $P < 10^{-4}$.

[e] $P < 10^{-4}$.

[f] $P < 10^{-5}$.

[g] $P < 0.02$.

independent segregation of the inclusion and tumor resistance phenotypes (data not shown).

DISCUSSION

Distal chromosome 1 carries one or more potent modifiers of liver cancer risk that account for most of the difference in tumor multiplicity between the C3H and B6 strains and all of the difference between BR and B6 males. Linkage analysis of crosses between the B6 and C3H or CBA strains indicate that a QTL, *Hcs7*, lies near *D1Mit33* at 82 cM. Our congenic analyses show that the C3H allele of *Hcs7* (*Hcs7*^{C3H}) is sufficient to confer susceptibility to the resistant B6 strain. The identification of *Hcs7* is based on both F_1 and congenic backcrosses, as well as F_2 intercrosses. Its location and independence were confirmed using N_{10} congenic lines. These methods exceed the most rigorous guidelines for QTL analysis promoted in a recently published white paper by the Complex Trait Consortium (2003). No other loci that are polymorphic in these crosses interact significantly with the *Hcs7* modifier. We have been unable to map *Hcs7* in several B6 × C3H recombinant inbred strains (Lee and Drinkwater 1995a), an observation that bears further study and might reveal interactions between recessive B6 and C3H alleles.

Hcs7^{C3H} has a 13- to 14-fold effect on liver tumor multiplicity in congenic males. The only known modifiers more potent than *Hcs7* in the liver are gender and growth hormone deficiency (Vesselinovitch and Mihailovich 1967; Vesselinovitch 1990; Bugni *et al.* 2001). Male mice are much more susceptible to liver tumorigenesis than females, and gonadectomy of either sex reduces this difference. Mutations in *Tfm* and *Ghrhr*, genes in the sex hormone and growth hormone pathways, confer 25- to 100-fold reductions in tumor multiplicity in carcinogen-treated mice (Kemp *et al.* 1989; Bugni *et al.* 2001). The *Hcs7* region contains no known component of these pathways. Accordingly, *Hcs7* appears to have an effect independent of sex: on a B6 background, *Hcs7*^{C3H} confers increased tumor multiplicity to a similar degree in both genders (Table 2).

The congenic (N_{10}) *Hcs7*^{C3H} modifier appears dominant in males and semidominant in females (Table 2). This difference is unlikely to reflect any real difference in the genders, because previous experiments suggest that *Hcs7* acts in a semidominant manner in males (Figure 1A; Drinkwater and Ginsler 1986). Rather, the apparent dominance in congenic males might reflect our inability to detect some tumors because of their high density in homozygotes under these conditions.

Among loci that have previously been mapped as liver cancer modifiers, only the BR *Hcf2* locus maps to the same chromosome as *Hcs7* (Gariboldi *et al.* 1993; Manenti *et al.* 1994; Lee *et al.* 1995; Poole and Drinkwater 1996). The possibility that *Hcs7* and *Hcf2* represent the same gene is supported by chimera analysis and the congenic data presented in Tables 1 and 2. In both C3H ↔ B6 chimeras and BR ↔ B6 chimeras, tumors develop mainly from the cells of the susceptible parent, suggesting that the predominant modifiers in C3H and BR act within hepatocytes (Condamine *et al.* 1971; Lee *et al.* 1991; Chiaverotti and Drinkwater 2003). In addition, both B6.C3H-Ch1 mice and B6.BR-Ch1 mice develop severalfold more tumors than do B6. However, the effect of the BR congenic region is less than that of the C3H region (6-fold *vs.* 13-fold). This ~2-fold difference might be explained by the presence of two (or more) polymorphic modifiers, only one of which is common to BR and C3H. Complexity in polymorphic modifier regions is frequent and might reflect the inheritance of linked gene families among inbred strains (Cormier *et al.* 2000; reviewed in Balmain 2002). Linked modifiers might also help explain the greater effect of the *Hcs7* region in the congenic lines than in the backcross and F_2 mice. In the congenics, the *Hcs7* locus might act additively with other minor loci in the region, while in the segregating crosses the linked genes would be separated by recombination at some frequency (results; Poole and Drinkwater 1996). The presence of a linked modifier might also explain the broad peak of the B6C3F_2 cross. Alternatively, the more proximal distribution of this intercross peak might reflect loci that depend on the carcinogen used to induce the tumors. We are resolving this issue by fine-structure mapping.

Much of the *Hcs7* region of chromosome 1 is ortholo-

gous to human chromosome 1q, which is amplified in about half of all tested hepatocellular carcinomas, independent of hepatitis status (Lin *et al.* 1999; Guan *et al.* 2000; Marchio *et al.* 2000; Tornillo *et al.* 2000; Wong *et al.* 2000; Zondervan *et al.* 2000). Chromosome 1q is also amplified in >50% of breast cancers and in 20–40% of tumors from a variety of tissues (Climent *et al.* 2002; Hislop *et al.* 2002; Sham *et al.* 2002). Our mapping suggests that *Hcs7* lies near *D1Mit33*, at 160 Mb on mouse chromosome 1. The region of mouse chromosome 1 orthologous to human chromosome 1q extends almost uninterrupted from 130 Mb to the end of the chromosome at 197 Mb. A minimal region frequently amplified in human HCC, 1q21–23 (Wong *et al.* 1999, 2000, 2001; Guan *et al.* 2000; Marchio *et al.* 2000), is orthologous to a subset of the congenic region close to *D1Mit33*, from ~168 Mb to 197 Mb (http://www.ensembl.org; October 2003). This region contains a number of intriguing genes common to mouse and man, including pre-B-cell leukemia transcription factor 1 (*Pbx1*), regulators of G-protein signaling 4 and 5 (*Rgs4*, *Rgs5*), TNF ligand superfamily member *Dedd*, the receptor tyrosine kinase *Ddr2*, activating transcription factor 6-α (*ATF6-α*), and Fas antigen ligand (*Tnfsf6*). Identification of the gene product(s) of the *Hcs7* locus is likely to lead to the identification of molecular targets for the prevention, early diagnosis, detection, or treatment of liver cancer.

The identity of *Hcs7* should also help explain the mechanism(s) of action of carcinogenic compounds identified in 2-year bioassays performed by the National Cancer Institute and the National Toxicology Program. About half of the compounds tested are carcinogenic to mice, rats, or both (Ashby and Tennant 1991). Of these, ~20% induce only liver tumors in $B6C3F_1$ mice. This response is likely to be mediated by *Hcs7*, which accounts for much of the susceptibility of $B6C3F_1$ mice to carcinogenesis by both DEN and ENU (Figure 1A; Tables 1 and 2). Key questions include whether a similar pathway is active in humans and whether humans carry a homolog of the susceptibility gene and a high frequency of functional variants.

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