Identification of Hepatocarcinogen-Resistance Genes in DBA/2 Mice

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Manuscript received March 16, 1994
Accepted for publication September 7, 1994

ABSTRACT

Male DBA/2J mice are ~20-fold more susceptible than male C57BL/6J mice to hepatocarcinogenesis induced by perinatal treatment with *N,N*-diethylnitrosamine (DEN). In order to elucidate the genetic control of hepatocarcinogenesis in DBA/2J mice, male BXD recombinant inbred, D2B6F$_1$ × B6 backcross, and D2B6F$_2$ intercross mice were treated at 12 days of age with DEN and liver tumors were enumerated at 32 weeks. Interestingly, the distribution of mean tumor multiplicities among BXD recombinant inbred strains indicated that hepatocarcinogen-sensitive DBA/2 mice carry multiple genes with opposing effects on the susceptibility to liver tumor induction. By analyzing D2B6F$_1$ × B6 backcross and D2B6F$_2$ intercross mice for their liver tumor multiplicity phenotypes and for their genotypes at simple sequence repeat marker loci, we mapped two resistance genes carried by DBA/2J mice, designated *Hcr1* and *-2*, to chromosomes *4* and *10*, respectively. *Hcr1* and *Hcr2* resolved the genetic variance in the backcross population well, indicating that these resistance loci are the major determinants of the variance in the backcross population. Although our collection of 100 simple sequence repeat markers allowed linkage analysis for ~95% of the genome, we failed to map any sensitivity alleles for DBA/2J mice. Thus, it is likely that the susceptibility of DBA/2J mice is the consequence of the combined effects of multiple sensitivity loci.

LABORATORY mouse strains differ markedly in susceptibility to hepatocarcinogenesis. Among the strains studied for liver tumor induction, C3H/HeJ (C3H) and CBA/J were the most susceptible to *N,N*-diethylnitrosamine (DEN) or *N*-ethyl-*N*-nitrosourea–induced hepatocarcinogenesis, whereas A/J, C57BL/6J (B6) and SWR/J were highly resistant to these carcinogens (DRINKWATER and BENNETT 1991). Because hepatocarcinogenesis in mice has been studied extensively as a model for multistage carcinogenesis, genetic analysis of this strain variation should provide important information regarding the mechanisms underlying tumor development. In fact, genetic analysis of segregating crosses between hepatocarcinogen-sensitive C3H and -resistant B6 or A/J mice identified several *Hcs* (*Hepatocarcinogen sensitivity*) loci (DRINKWATER and GINSLER 1986; BENNETT *et al.* 1993; GARIBOLDI *et al.* 1993) that influence the multiplicity or growth rates of preneoplastic or neoplastic hepatic lesions (HANIGAN *et al.* 1988; DRAGANI *et al.* 1991).

Our laboratory has also been interested in hepatocarcinogenesis in DBA/2J (D2) mice because the sensitivity of this strain depends on the timing and the type of carcinogenic treatment. A comparative study from our laboratory indicated that, if they were treated with DEN at 12 days of age, D2 mice developed ~20-fold more liver tumors than hepatocarcinogenesis-resistant B6 mice (DRINKWATER and BENNETT 1991; BENNETT *et al.* 1992). This level is 70% of that of the best characterized hepatocarcinogen-sensitive mouse strain, C3H. D2B6F$_1$ and B6D2F$_1$ mice are only slightly less sensitive than the parental D2 mice, indicating that this sensitivity is inherited in an autosomal, semidominant manner (BENNETT *et al.* 1992). The high sensitivity of infant D2 mice to DEN was unexpected because DIWAN *et al.* (1986) had reported previously that DEN treatment of 5-week-old male D2 mice resulted in a yield of liver tumors that was more similar to that for B6 mice than to C3H mice. D2 mice also have a very low incidence of spontaneous hepatomas (1.5%) (SMITH *et al.* 1973), whereas the incidence for C3H mice is extremely high (up to 100%) (GRASSO and HARDY 1975).

A further intriguing feature of hepatocarcinogenesis in D2 mice was revealed by our recent study of the growth kinetics of preneoplastic hepatic lesions in male D2 mice initiated with a perinatal injection of DEN (BENNETT *et al.* 1992). Under these experimental conditions, preneoplastic hepatocellular lesions in male D2 and C3H mice showed similar high growth rates relative to B6 lesions. However, when the numbers of lesions were compared among the strains, C3H mice developed a fivefold greater number of lesions than D2 or B6 mice.

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These results led us to suggest that D2 mice may be relatively resistant to the induction of the early events that give rise to preneoplastic lesions, but that, once they are induced, these lesions proliferate more rapidly and are more likely to progress to neoplasms than those in B6 mice (PITOT *et al.* 1987; LEE *et al.* 1989a; BENNETT *et al.* 1992).

The biological complexity of hepatocarcinogenesis in D2 mice indicated that genetic control of tumor development in this strain might also be complex. In the present study, we attempted to dissect genetically the hepatocarcinogen-sensitivity of D2 mice by analyzing BXD recombinant inbred (RI) mice, and D2B6F$_1$ × B6 backcross and D2B6F$_2$ intercross mice treated with DEN at 12 days of age. Although D2 mice are very sensitive to this protocol, the striking result was that D2 mice carry two genes that reduce significantly liver tumor multiplicity. The two loci were mapped to chromosomes *4* and *10*, respectively, and each locus was associated with a 50 to 70% reduction in mean tumor multiplicity in heterozygous animals. However, after scanning >95% of the mouse genome, we were not able to discern a locus for which the D2 allele was associated with a significant increase in liver tumor multiplicity. We hypothesize that the sensitivity of D2 mice to perinatal treatment with DEN is determined by the two resistance loci that we mapped and by multiple sensitivity loci not identified in this study. The successful mapping of the two resistance loci should provide insight into the paradoxical features of hepatocarcinogenesis in the D2 mouse.

MATERIALS AND METHODS

Animals: The mice used in this study were bred in the laboratory from stocks of C57BL/6J (B6), DBA/2J (D2) and BXD RI strains of mice purchased from the Jackson Laboratory (Bar Harbor, ME). Among the RI strains available, we excluded BXD-9, -14, -20 and -22 because of their poor fertility. D2B6F$_1$ × B6 backcross mice were generated by mating male B6 mice with female D2B6F$_1$ mice (which are the F$_1$ generation of a D2 mother and a B6 father). D2B6F$_2$ mice were obtained by intercrossing male and female D2B6F$_1$ mice. Animals were housed in plastic cages on corn cob bedding (Bed-O'Cobs, Anderson Cob Division, Maumee, OH), fed Wayne Breeder Blox (Continental Grain Co., Chicago, IL), and allowed free access to food and water.

Induction of Liver Tumors: At 12 days of age, each male mouse was injected i.p. with DEN (Eastman Kodak Co., Rochester, NY) dissolved in sterile trioctanoin (Pfaltz and Bauer, Inc., Stamford, CT) (0.01 ml/g body wt) at a dose level of 0.05 μmol/g body wt. At 32 weeks of age, mice were killed by CO_2 asphyxiation and the numbers of tumors on the liver surface >1 mm in diameter were recorded. The spleens of D2B6F$_1$ × B6 backcross and D2B6F$_2$ intercross mice were collected and frozen on dry ice as a source from which to extract genomic DNA.

Statistical comparisons of mean tumor multiplicity data were performed by the two-sided Wilcoxon rank sum test (LEHMANN 1975).

Genotypic analysis of simple sequence repeat (SSR) loci by polymerase chain reaction (PCR): The spleen DNA of each backcross mouse was extracted with proteinase K-phenol by a standard method (SAMBROOK *et al.* 1989) and used as templates for the genotyping of SSR markers by PCR (AITMAN *et al.* 1991; DIETRICH *et al.* 1992a). We selected 100 informative SSR markers (AITMAN *et al.* 1991; DIETRICH *et al.* 1992b) with an average spacing of ~15 cM (Table 1). At least four markers were analyzed for each chromosome. The largest recombination fraction between adjacent markers observed in our backcross was 0.30 in the *D16Mit9–D16Mit4* interval and the second largest ones were 0.22 in the *D6Mit10–D6Mit15* and *D9Mit8–D9Mit20* intervals. Our collection of markers allowed us to cover ~95% of the mouse genome, assuming that each marker would detect linkage to a quantitative trait locus up to 15 cM distant (DARVASI *et al.* 1993; HILLYARD *et al.* 1993). All the PCR primer pairs for SSR markers were purchased from Research Genetics (Huntsville, AL) except for the *Zp3* marker, which was obtained by custom synthesis (Oligos, etc., Wilsonville, OR) according to the published primer sequences (AITMAN *et al.* 1991; DIETRICH *et al.* 1992a). PCR conditions were essentially as described by DIETRICH *et al.* (1992a) using *Taq* DNA polymerase from Promega (Madison, WI) and a DNA thermal cycler from Perkin-Elmer Cetus (Norwalk, CT), except that the number of cycles was increased to 50 and the primers were not radioactively labeled. A 20-μl aliquot of each PCR reaction was applied to a 7% nondenaturing polyacrylamide gel and electrophoresed. The gel was then stained with ethidium bromide. Under ultraviolet illumination, PCR products were visualized, photographed and scored for the presence of B6- and D2-specific alleles according to the published strain-specific sizes of the bands (AITMAN *et al* 1991; DIETRICH *et al.* 1992a).

Linkage analysis for liver tumor sensitivity genes in D2B6F$_1$ × B6 backcross mice: From a total of 71 backcross mice, only 15 each at the low (tumor multiplicity 0–3) and high (tumor multiplicity 29–71) extremes of tumor multiplicity were genotyped for 100 SSR markers as an initial screen. For markers linked to a gene with an effect on tumor multiplicity, we would expect the proportions of sensitive and resistant animals to be significantly different by Fisher's exact test (SOKAL and ROHLF 1981) for homozygotes and heterozygotes at the marker locus. If a marker showed a possible linkage (*i.e.*, $P < 0.05$) in this primary screen, the remaining 41 backcross mice were genotyped at the marker locus and the mean tumor multiplicities for homozygotes and heterozygotes were compared by a two-tailed Wilcoxon rank sum test (LEHMANN 1975) as the secondary screen. If the test marker still showed a significant difference ($P < 0.05$) in tumor multiplicity, all mice were genotyped for all the markers located on the same chromosome. Finally, our criterion for linkage of a marker locus to a susceptibility gene was that a P value less than 0.0005 was obtained by the two-sided Wilcoxon rank sum test. This per marker significance level was set by simply dividing the desired experiment-wise significance level of $P = 0.05$ by the number of markers we analyzed (100 markers). This approach is highly conservative because of the linkage within subsets of the marker loci (DARVASI *et al.* 1993). Although this three-step approach reduced our task for genotypic analysis by more than a half, it should be noted that quantitative trait loci with small phenotypic effects might have been missed in the earlier steps. All of the available genotype data were also analysed by the Mapmaker/QTL program described by LANDER and BOTSTEIN (1989) to obtain LOD scores for linkage. For this analysis, tumor multiplicity data were transformed by an equation proposed by ANSCOMBE (1948) to improve the fit of negative binomial data to the normal distribution (DRINKWATER and KLOTZ 1981).

TABLE 1

SSR markers used in this study

Locus	Chromosome (cM)[a]	Locus	Chromosome (cM)	Locus	Chromosome (cM)
D1Mit1	*1* (6)	*D6Rck2*	*6* (4)	*D12Mit1*	*12* (4)
D1Mit70	*1* (16)	*D6Mit16*	*6* (22)	*D12Mit2*	*12* (19)
D1Mit5	*1* (37)	*D6Mit10*	*6* (45)	*D12Mit3*	*12* (30)
D1Mit11	*1* (63)	*D6Mit15*	*6* (69)	*D12Mit7*	*12* (48)
D1Mit30	*1* (78)			*D12Mit8*	*12* (62)
D1Mit102	*1* (80)	*D7Mit21*	*7* (9)		
D1Mit16	*1* (89)	*D7Nds5*	*7* (26)	*D13Mit3*	*13* (4)
D1Mit17	*1* (113)	*D7Nds2*	*7* (35)	*D13Mit13*	*13* (23)
		D7Mit7	*7* (50)	*D13Mit30*	*13* (39)
D2Mit6	*2* (10)	*D7Mit12*	*7* (71)	*D13Mit35*	*13* (62)
D2Mit7	*2* (28)				
D2Mit9	*2* (38)	*D8Mit4*	*8* (13)	*D14Mit1*	*14* (1)
D2Mit14	*2* (49)	*D8Mit8*	*8* (36)	*D14Mit5*	*14* (32)
D2Mit21	*2* (67)	*D8Mit11*	*8* (47)	*D14Mit7*	*14* (54)
D2Mit52	*2* (85)	*D8Mit88*	*8* (62)	*D14Mit75*	*14* (66)
		D8Mit56	*8* (78)		
D3Mit54	*3* (5)			*D15Mit13*	*15* (1)
D3Mit21	*3* (15)	*D9Mit65*	*9* (5)	*D15Mit5*	*15* (18)
D3Mit12	*3* (39)	*D9Mit22*	*9* (23)	*D15Mit31*	*15* (36)
D3Mit42	*3* (44)	*D9Mit21*	*9* (30)	*D15Mit16*	*15* (62)
D3Mit17	*3* (53)	*D9Mit8*	*9* (42)		
D3Mit19	*3* (69)	*D9Mit20*	*9* (60)	*D16Mit9*	*16* (3)
		D9Mit18	*9* (70)	*D16Mit4*	*16* (27)
D4Mit41	*4* (12)			*D16Mit5*	*16* (35)
D4Mit17	*4* (25)	*D10Mit3*	*10* (15)	*D16Mit6*	*16* (48)
D4Mit9	*4* (32)	*D10Mit40*	*10* (19)		
D4Mit32	*4* (44)	*D10Mit15*	*10* (24)	*D17Mit26*	*17* (2)
D4Mit12	*4* (49)	*D10Mit10*	*10* (52)	*D17Mit10*	*17* (21)
D4Mit16	*4* (51)	*D10Mit14*	*10* (71)	*D17Mit3*	*17* (36)
D4Mit71	*4* (54)			*D17Mit41*	*17* (49)
D4Mit13	*4* (65)	*D11Mit2*	*11* (5)		
D4Mit14	*4* (68)	*D11Mit19*	*11* (12)	*D18Mit19*	*18* (1)
		D11Mit20	*11* (21)	*D18Mit17*	*18* (15)
D5Mit1	*5* (3)	*D11Mit4*	*11* (38)	*D18Mit33*	*18* (30)
D5Mit11	*5* (19)	*D11Nds7*	*11* (66)	*D18Mit4*	*18* (39)
D5Nds2	*5* (30)				
D5Mit10	*5* (40)			*D19Mit6*	*19* (1)
D5Mit24	*5* (49)			*D19Mit16*	*19* (14)
Zp3	*5* (65)			*D19Mit11*	*19* (28)
D5Mit99	*5* (80)			*D19Mit1*	*19* (43)

[a] The chromosome and map position relative to the most proximal known SSR marker are indicated for each locus (DIETRICH *et al.* 1992a,b; supplemented by additional markers in Whitehead Institute/MIT Center for Genome Research, Genetic Map of the Mouse, Database Release 10/93).

Confirmation of putative hepatocarcinogen sensitivity loci by linkage analysis using D2B6F$_2$ intercross mice: Forty-six D2B6F$_2$ intercross mice were genotyped at the marker loci that showed significant or suggestive linkage to a quantitative trait locus in the analysis of the backcross mice. The mean tumor multiplicities for the three possible marker genotypes were compared by a one-sided Jonckheere-Terpstra test (LEHMANN 1975). In this nonparametric test, the null hypothesis that the tumor multiplicities for the three genotypes were equal was tested against the alternative that the multiplicity increased or decreased with the dosage of the D2 allele, depending on the positive or negative effect of the locus inferred from the backcross analysis. Using the two-sided P values obtained for the backcross and intercross mice by the Wilcoxon rank sum test and the Jonckheere-Terpstra test, respectively, combined P values were calculated for each locus according to the method of Fisher (SOKAL and ROHLF 1981) in order to evaluate the overall significance of linkage under the null hypothesis that genotypes at the marker locus do not influence liver tumor multiplicity. The significance level for linkage was set at $P = 0.0005$ as described above. The LOD score analysis was also performed by using the Mapmaker/QTL program as already described.

Analysis for loss of heterozygosity in D2B6F$_1$ liver tumors: Male D2B6F$_1$ mice were injected i.p. with 0.05 μmol/g body wt of DEN at 12 days of age as described above. Liver tumors were collected at 32 weeks of age by careful dissection from surrounding normal tissue, and genomic DNAs were

TABLE 2

DEN-initiated liver tumors in BXD RI mice

Strain	No. of mice	Mean liver tumor multiplicity[a]
BXD-11	40	63 ± 3[b]
BXD-19	33	43 ± 3
BXD-5	28	22 ± 2
BXD-16	33	22 ± 2
BXD-8	32	18 ± 3
BXD-18	31	16 ± 2
BXD-24	35	15 ± 2
BXD-27	15	14 ± 3
BXD-21	31	14 ± 2
BXD-2	37	14 ± 2
BXD-25	36	12 ± 2
BXD-31	36	8.6 ± 1.3
BXD-13	11	8.2 ± 3.6
BXD-32	28	8.1 ± 1.5
BXD-23	32	5.8 ± 1.2
BXD-28	30	5.6 ± 1.0
BXD-30	13	3.5 ± 1.0
BXD-1	29	2.7 ± 0.9
BXD-29	32	2.3 ± 0.6
BXD-12	31	1.7 ± 0.4
BXD-6	33	1.3 ± 0.4
BXD-15	34	0.06 ± 0.04[c]
DBA/2J	31	31 ± 4
C57BL/6J	28	1.6 ± 0.5

Male mice from each strain were treated at 12 days of age with *N,N*-diethylnitrosamine and the number of induced liver tumors was determined at 32 weeks of age.

[a] Values are means ± SE.

[b] Significantly different from the DBA/2J value by Wilcoxon rank sum test ($P < 0.00001$ for BXD-11; $P < 0.05$ for BXD-19).

[c] Significantly different from the C57BL/6J value by Wilcoxon rank sum test ($P < 0.0001$).

extracted. Loss of heterozygosity at selected SSR marker loci was determined by analysis using the liver tumor DNAs as templates.

RESULTS

Sensitivities of BXD RI strains to hepatocarcinogenesis induced by perinatal treatment with DEN: The mean tumor multiplicities of the BXD RI strains and the parental D2 and B6 strains are shown in Table 2. Two RI strains, BXD-11 and -19, had exceptionally high mean tumor multiplicities of 63 ± 3 and 43 ± 3 (mean ± SE), respectively. Both values were significantly greater than the value of 31 ± 4 obtained for the parental D2 strain ($P < 0.00001$ for BXD-11, $P < 0.05$ for BXD-19). This result indicated that D2 mice may carry significant resistance alleles that were not present in BXD-11 and -19 mice, in addition to sensitivity alleles shared by the three strains. Consistent with this hypothesis, there was also one strain, BXD-15, which had a significantly lower mean tumor multiplicity of 0.06 ± 0.04 relative to the mean tumor multiplicity of 1.6 ± 0.5 for the parental B6 strain ($P < 0.0001$). Except for the three extreme strains, BXD-11, -19 and -15, the RI strains showed resistant or intermediate phenotypes within the range displayed by the parental D2 and B6 mice, and no clustering of the phenotype was obvious.

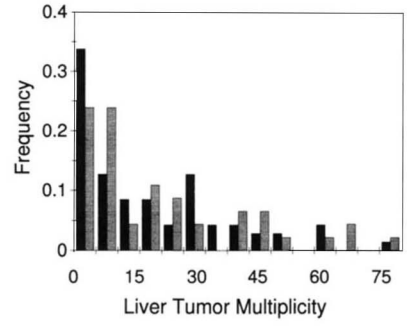

FIGURE 1.—Distribution of liver tumor multipl cities in the 71 D2B6F$_1$ × B6 backcross (■) and 46 D2B6F$_2$ intercross (▨) mice. Male mice were treated with *N,N*-diethylnitrosamine at 12 days of age and liver tumors were enumerated at 32 weeks of age.

Linkage analysis of perinatal, DEN-induced hepatocarcinogenesis using D2B6F$_1$ × B6 backcross mice: The distribution of liver tumor multiplicities for the 71 backcross mice treated with DEN at 12 days of age is shown in Figure 1. The apparently nonnormal distribution of tumor multiplicities is consistent with the segregation of loci that control susceptibility to tumor induction.

After the second screen for linkage (see METHODS), our results indicated that D2 alleles of *Zp3* (chromosome *5*) and *D12Mit3* (chromosome *12*) were associated with sensitivity to liver tumor induction, and those of *D4Mit9, -12, -13,* and *-14* (chromosome *4*) and *D10Mit10,* and *-15* (chromosome *10*) were linked to resistance genes. Thus, all 71 backcross mice were genotyped for all markers on chromosomes *4, 5, 10* and *12*. The mean tumor multiplicities as a function of the genotypes at these loci are summarized in Table 3. Significance levels under the threshold of 0.0005 were obtained only for the chromosome *4* loci. The most significant result was obtained for the *D4Mit31* locus ($P = 0.00021$). The D2 allele at this locus was associated with approximately about a 60% reduction in the number of liver tumors. We designated this resistance locus as *Hcr1* (*Hepatocarcinogen resistance*). The locus *D10Mit15* also gave a marginally signficant *P* value of 0.00057. The D2 allele at this locus was associated with about a 50% reduction in the tumor number. Therefore, we tentatively named this locus *Hcr2*. The LOD scores calculated by using the Mapmaker/QTL program at *D4Mit31* and *D10Mit15* were 3.1 and 3.0, respectively. For marker loci located on chromosomes *5* and *12*, the best *P* values were much larger than the required

TABLE 3

Summary of the linkage analyses

Chromosome	Locus[a]	Backcross (n = 71) Genotype B/B^b	Backcross (n = 71) Genotype D/B^b	Backcross P	Backcross LOD score	Intercross (n = 46) Genotype B/B^b	Intercross B/D or D/B	Intercross D/D	Intercross P	Intercross LOD score	Combined P^c	Total LOD score
4	*D4Mit17*	20 ± 3 (35)	14 ± 3 (36)	0.033	1.3	18 ± 5 (9)	25 ± 4 (24)	11 ± 5 (13)	0.057	1.5	0.025	2.8
	D4Mit9	24 ± 3 (33)	11 ± 2 (38)	0.0017	2.3	25 ± 6 (11)	24 ± 4 (24)	6.1 ± 2.3 (11)	0.0024	3.3	0.0001	5.6
	D4Mit31	26 ± 3 (32)	10 ± 2 (39)	0.00021	3.1	24 ± 5 (13)	22 ± 4 (22)	10 ± 4 (11)	0.015	2.2	0.00009	5.3
	D4Mit12	25 ± 3 (34)	10 ± 2 (37)	0.00057	2.7	26 ± 5 (17)	20 ± 4 (18)	10 ± 4 (11)	0.010	2.2	0.00013	4.9
	D4Mit16	24 ± 4 (36)	9.9 ± 1.7 (35)	0.00079	2.6	27 ± 6 (16)	19 ± 4 (19)	10 ± 4 (11)	0.0059	2.2	0.00011	4.8
	D4Mit71	24 ± 3 (40)	8.6 ± 1.7 (31)	0.00045	2.7	27 ± 6 (16)	20 ± 4 (18)	9.9 ± 3.8 (12)	0.0049	2.2	0.00008	4.9
	D4Mit13	25 ± 3 (33)	9.9 ± 1.6 (38)	0.00069	2.3	26 ± 6 (16)	18 ± 4 (17)	15 ± 5 (13)	0.035	0.8	0.00050	3.1
	D4Mit14	26 ± 3 (32)	9.7 ± 1.6 (39)	0.00037	2.6	25 ± 6 (16)	19 ± 4 (17)	15 ± 5 (13)	0.072	0.8	0.00055	3.4
10	*D10Mit3*	20 ± 3 (34)	15 ± 3 (37)	0.041	1.2	26 ± 8 (9)	19 ± 4 (26)	16 ± 5 (11)	0.15	0.3	0.065	1.5
	D10Mit40	22 ± 3 (31)	13 ± 3 (40)	0.0044	2.1	31 ± 8 (9)	18 ± 3 (28)	14 ± 5 (9)	0.029	0.5	0.00025	2.6
	D10Mit15	24 ± 3 (33)	11 ± 2 (38)	0.00057	3.0	32 ± 8 (9)	18 ± 3 (29)	13 ± 6 (8)	0.0089	1.3	0.00013	4.3
	D10Mit10	24 ± 3 (32)	11 ± 2 (39)	0.00062	2.9	29 ± 7 (11)	17 ± 3 (21)	17 ± 5 (14)	0.043	0.9	0.00060	3.8
	D10Mit14	20 ± 3 (34)	15 ± 2 (35)	0.41	0.1	27 ± 6 (13)	16 ± 3 (22)	20 ± 7 (11)	0.086	0.6	0.25	0.7
5	*D5Mit24*	16 ± 2 (41)	19 ± 3 (30)	0.78	0.0	20 ± 6 (9)	18 ± 3 (26)	24 ± 7 (11)	0.41	−0.15		−0.15
	Zp3	13 ± 2 (39)	23 ± 4 (32)	0.017	1.2	17 ± 8 (6)	18 ± 3 (28)	25 ± 7 (12)	0.20	0.2		1.4
	D5Mit99	13 ± 2 (37)	22 ± 3 (34)	0.048	0.9	19 ± 9 (6)	18 ± 3 (27)	24 ± 7 (13)	0.28	0.2		1.1
12	*D12Mit2*	13 ± 3 (26)	19 ± 3 (45)	0.042	1.0	16 ± 6 (10)	22 ± 4 (22)	19 ± 5 (14)	0.45	0.0		1.0
	D12Mit3	9.3 ± 2 (23)	21 ± 3 (48)	0.0063	1.7	18 ± 6 (10)	16 ± 3 (18)	26 ± 5 (18)	0.24	0.3		2.0
	D12Mit7	13 ± 3 (24)	19 ± 3 (47)	0.098	0.6	17 ± 4 (7)	19 ± 3 (22)	22 ± 5 (17)	0.45	−0.2		0.4

The mean tumor multiplicities and standard errors of backcross and intercross mice are shown as a function of their genotypes at selected marker loci; no. of mice in parentheses. The significance levels (P values) for the comparisons of these groups by the Wilcoxon rank sum test (backcross) or Jonckheere-Terpstra test (intercross) are shown as a test for linkage of the markers to a locus influencing tumor induction. The LOD scores for the presence of a quantitative trait locus at each marker locus were computed using the Mapmaker QTL program.

[a] Loci are in order from centromeric to telomeric.

[b] B = C57BL/6J allele, D = DBA/2J allele.

[c] A joint significance level for the difference between genotypes in liver tumor multiplicity was obtained by combining the results for the two crosses by the method of Fisher (SOKAL and ROHLF 1981).

significance level of 0.0005; however, in both cases, the D2 alleles were associated with about twofold increases in the tumor number.

Analysis of D2B6F$_2$ intercross mice for confirmation of putative hepatocarcinogen sensitivity loci: In order to characterize further the susceptibility loci implicated by the backcross analysis, we determined the genotypes of 46 F$_2$ intercross mice for 19 SSR markers located on chromosomes *4*, *10*, *5* and *12* (Table 3). The distribution of liver tumor multiplicities in these animals is shown in Figure 1. The data obtained for the intercross mice were consistent with the existence of two liver

tumor resistance genes on chromosomes *4* and *10*. For the *Hcr1* locus on chromosome *4*, the most significant results were for the markers *D4Mit9* ($P = 0.0024$) and *D4Mit71* ($P = 0.0049$). At both the loci, homozygous D2 alleles were associated with a 60 to 70% reduction in mean liver tumor multiplicity compared with homozygous B6 alleles. The mean tumor multiplicities for mice heterozygous for either chromosome *4* locus were intermediate to those for the two homozygous genotypes but were not significantly different from those for mice homozygous for the B6 alleles. For the *Hcr2* locus on chromosome *10*, the best evidence for linkage was obtained with *D10Mit15* ($P = 0.0089$). At this locus, the homozygous D2 alleles were also associated with a 60 to 70% reduction in mean tumor multiplicity relative to the homozygous B6 genotype. The relative mean tumor multiplicities for the three possible genotypes were consistent with a semidominant effect of the *Hcr2* resistance allele. The LOD scores at *D4Mit9*, *D4Mit71* and *D10Mit15*, calculated using the Mapmaker/QTL program without assumption of the mode of inheritance, were 3.3, 2.2 and 1.3, respectively. Analysis of F_2 mice for markers on chromosomes *5* and *12*, for which the backcross analysis demonstrated weak linkage to D2 sensitivity alleles, failed to confirm the existence of quantitative trait loci influencing liver tumor induction on these chromosomes.

In order to test the overall significance of tests for linkage of the chromosome *4*, *5*, *10* and *12* markers to quantitative trait loci affecting liver tumor induction, the results of the backcross and intercross analyses were combined for each marker locus by summing the LOD scores for the two crosses or using the method of Fisher to derive a joint significance level (Table 3). Several marker loci on chromosomes *4* and *10* showed combined significance levels well below the threshold of 0.0005 and total LOD scores well above the conventional threshold of 3. Therefore, we concluded that there was strong evidence for the existence of loci that suppress liver tumor development on these chromosomes. On the other hand, the weak linkage between loci on chromosomes *5* and *12* and D2 alleles that enhanced liver tumor induction was not borne out by further analysis.

Resolution of the backcross distribution according to *Hcr* genotypes: The distribution of tumor multiplicities for the backcross mice was reconstructed according to the genotypes at *D4Mit31* and *D10Mit15*, which gave the best *P* values and LOD scores on chromosomes *4* and *10*, respectively, in order to see how well the *Hcr* genotypes resolved the segregation pattern. The results are summarized in Table 4. As expected, the liver tumor multiplicities were influenced largely by the dosage of the resistance genes on chromosomes *4* and *10*. This result indicates that the *Hcr* loci are indeed major determinants of the segregation of tumor multiplicity. The backcross mice with B6 alleles at both the loci had the greatest mean tumor multiplicity of 32 ± 4 (SE). This value was significantly higher ($P < 10^{-6}$) than the mean tumor multiplicity of the backcross mice with D2 alleles at both loci (7.5 ± 1.9). Comparison of the mean tumor multiplicities of the mice with the parental genotypes with those heterozygous for each locus indicated that the effects of the *Hcr1* and *-2* resistance alleles on liver tumor induction were additive in this cross. Because of the large number of potential genotypes and the small number of animals representing each genotype, we have not performed a similar analysis of the data from the intercross.

TABLE 4

Liver tumor multiplicities of the backcross mice according to the genotypes at *D4Mit31* and *D10Mit15*

Genotype[a] at		
D4Mit31	*D10Mit15*	Tumor No.
B/B	*B/B*	32 ± 4 (18)
B/B	*D/B*	18 ± 5 (14)
D/B	*B/B*	14 ± 3 (15)
D/B	*D/B*	7.5 ± 1.9 (24)[b]

The markers *D4Mit31* and *D10Mit15* are linked closely to the *Hcr1* and *Hcr2* loci inferred from the genetic analysis of susceptibility to liver tumor induction. The data shown are the mean tumor multiplicities for backcross mice with the four possible genotypes at the two markers with no. of mice in parentheses.

[a] *B* = C57BL/6J allele, *D* = DBA/2J allele.

[b] Significantly different by the Wilcoxon rank sum test from the value for the mice homozygous for *B* alleles at both loci ($P < 10^{-6}$).

Evaluation of the effects of the *Hcr* loci in BXD RI mice: The BXD RI strain distribution patterns are known for a number of loci located near the *Hcr1* locus (DIETRICH *et al.* 1992a; TAYLOR 1981; B. TAYLOR, personal comunication). Among the 11 loci for which information is available, *Xmmv-23*, which is located slightly distal to *D4Mit16* showed suggestive linkage to *Hcr1*. The average of the mean liver tumor multiplicity for BXD RI strains with D2 alleles at *Xmmv-23* was 8.0 (Table 2; strains BXD-8, -18, -21, -25, -13, -32, -23, -28, -30, -1, -12 and -15). On the other hand, the average of those for the strains with B6 alleles at *Xmmv-23* was 23 (Table 2; BXD-11, -19, -5, -16, -24, -27, -2, -31 and -29). This significant result ($P < 0.009$) may indicate that *Hcr1* is tightly linked to *Xmmv-23*. *Xmmv-23* lies in the middle of the one LOD support interval indicated by the linkage analysis.

Unfortunately, the RI strain distribution patterns have been reported for only a few loci near *Hcr2*, making it difficult to evaluate the effect of the *Hcr2* locus in the RI mice. *D10Mit3*, *D10Mit15* and *D10Mit10*, for which the distribution patterns are known (DIETRICH *et*

al. 1992a), did not show any suggestive linkage to *Hcr2,* probably as a result of frequent recombinations in the RI mouse genomes (TAYLOR 1981).

Loss of heterozygosity at *Hcr* loci of D2B6F$_1$ mouse liver tumors: Because loss of heterozygosity at tumor suppressor loci frequently occurs in human neoplasms, including hepatocellular carcinomas (BUETOW *et al.* 1989; SIMON *et al.* 1991), we searched for possible loss of the chromosomal region surrounding the *Hcr* loci in D2B6F$_1$ mouse liver tumors by analysis of the tumor cell genotypes at the *D4Mit9, D4Mit31, D4Mit12, D4Mit13, D10Mit3* and *D10Mit15* loci. Of 10 liver tumors analyzed, only one tumor showed a loss of heterozygosity at the *D10Mit15* locus. In this case, the D2 allele was lost (data not shown).

DISCUSSION

We have successfully mapped two loci for which alleles carried by D2 mice inhibit liver tumor induction by perinatal treatment with DEN. The two loci are located on chromosomes *4* and *10* and are designated *Hcr1* and *-2,* respectively. The regions where *Hcr1* and *-2* lie include some cancer-related genes including the *c-jun, L-myc* and *c-fgr* proto-oncogenes on chromosome *4,* or the *c-fyn* and *c-ros-1* proto-oncogenes on chromosome *10* (ABBOTT *et al.* 1992; TAYLOR *et al.* 1992). The *c-jun* oncogene was shown recently to be essential for hepatogenesis in the mouse (HILBERG *et al.* 1993). Three genes that act dominantly to inhibit tumor development at other tissue sites have been mapped to the same region of chromosome *4* that carries *Hcr1.* The *Mom-1* locus, located near *D4Mit13,* suppresses the development of intestinal tumors in mice that carry the *Min* mutation of the mouse *Apc* gene (DIETRICH *et al.* 1993). POTTER and co-workers have identified two loci, *Pct-1* and *Pct-2,* which map near *D4Mit12* and *D4Mit14,* respectively, that inhibit the induction of plasmacytomas by pristane (POTTER *et al.* 1994).

Comparative studies of the mouse and human chromosomal maps indicate that the human counterparts of *Hcr1* and *-2* are located on human chromosomes *1* and *6,* respectively (NADEAU *et al.* 1992). While some studies have shown that chromosomal aberrations, typically loss of heterozygosity for tumor suppressor gene loci, are not common for those chromosomes in human hepatocellular carcinomas (BUETOW *et al.* 1989; ZHANG *et al.* 1990), SIMON *et al.* (1991) found that 10 of 12 human liver tumors contained gross deletions or loss of heterozygosity on chromosome *1p.* Although we detected loss of the D2 allele at *D10Mit15,* a locus linked to *Hcr2,* in 1 of 10 DEN-initiated D2B6F$_1$ mouse liver tumors, the low frequency of loss indicates that it may be a nonspecific event unrelated to tumor development. However, a recent study by NISHIMORI *et al.* (1994) demonstrated that immortalized liver epithelial cell lines derived from C3H mouse hepatocytes invariably carried deletions in chromosome *4.* In collaboration with that group, we have found that similar cell lines derived from C3B6F$_1$ mice often demonstrated loss of heterozygosity in the region of chromosome *4* containing the *Hcr1* locus (G.-H. LEE, N. DRINKWATER, H. NISHIMORI and K. OGAWA, unpublished data).

Because male D2 mice are ~20-fold more susceptible than male B6 mice to the protocol used in this study to induce liver tumors (DRINKWATER and BENNETT 1991; BENNETT *et al.* 1992), it is puzzling that we were able to map only resistance alleles from D2 mice. Clearly, D2 mice must possess some sensitivity locus or loci to account for their high susceptibility relative to B6 mice.

It is unlikely that a single susceptibility locus is responsible for the high sensitivity of D2 mice to hepatocarcinogenesis and that we failed to detect it in our linkage analysis. The largest recombination fraction between adjacent marker loci analyzed in the backcross was 0.30, and ~95% of the autosomal mouse genome was located within a 15-cM distance from one of the markers selected. In our initial screen for quantitative trait loci, *D4Mit9* and *D4Mit14,* which showed a recombination fraction of 0.33, and all of the markers between them showed positive results ($P < 0.05$ by Fisher's exact test). Thus, loci having greater effects on the genetic variance than the *Hcr1* locus should not have been missed in the first screening. Based on the analysis by DARVASI *et al.* (1993) of the effect of marker spacing on the power of tests for detecting linkage between marker and quantitative trait loci, the experiment we performed, using an average distance of 20 cM between markers, was 90% as powerful as the hypothetical case of an infinitely dense collection of markers. The data for the BXD RI mouse strains also support the conclusion that more than one sensitivity locus is carried by D2 mice. If a single, predominant D2 sensitivity locus existed, about half of the BXD RI strains would be expected to be as sensitive as or more sensitive than the parental D2 strain as a result of the segregation of *Hcr1, -2* and the notional strong sensitivity locus. However, most of the RI strains (18 of 22) showed significantly lower mean tumor multiplicities than that of the parental D2 mice ($P < 0.05$ by the two-sided Wilcoxon rank sum test). This observed ratio (4:18) was significantly different from the predicted ratio (11:11) by a chi-square test ($P < 0.005$).

We believe that it is more likely that the high susceptibility of D2 mice to liver tumor induction results from the combined effects of multiple sensitivity genes. In this model, D2 mice would carry sensitivity alleles at multiple loci, each with relatively small individual effects; together the D2 alleles are able to overcome the resistance effects of *Hcr1* and *-2.* In the case that these genes showed epistatic interactions, the presence of as few as three sensitivity alleles in D2 mice would impede

seriously our efforts to detect them in our linkage studies. The effective number of loci contributing to a quantitative, phenotypic difference between two inbred strains may be estimated from the variance of the backcross or intercross and the means and variances of the parental and F_1 animals using formulas derived by WRIGHT (1968). Using this approach to analyze our backcross and intercross data, we estimate the number of effective loci controlling susceptibility to be 2.8 and 3.0, respectively. These values are clearly underestimates given that WRIGHT's formula is based on the assumptions that the controlling loci are all equal in their effect on the phenotype, that they act additively, and that all of the resistance alleles are carried by the resistant parent.

It is extremely difficult to map sensitivity loci when cancer susceptibility is controlled by multiple genes or genes with epistatic interactions (DEMANT *et al.* 1989). Detection of linkage for any of the multiple sensitivity loci that we postulate to be carried by the D2 mice would require analysis of a very large number of backcross or intercross animals. Recently, MOEN *et al.* (1992) reported that their approach using recombinant congenic mice enabled them to map an individual colon cancer susceptibility locus in STS/A mice. The development of recombinant congenic strains, in which the confounding effects of the *Hcr* loci would be eliminated, would allow us to map the susceptibility genes carried by D2 mice more readily. Alternatively, we could attempt to map D2 susceptibility genes in crosses between B6 mice and the most sensitive recombinant inbred, BXD-11.

The D2 strain of mice has generally been considered to be resistant to hepatocarcinogenesis, because it has a very low incidence of spontaneous hepatomas (SMITH *et al.* 1973) and is resistant to postweaning injection of DEN (DIWAN *et al.* 1986). The shift from neonatal sensitivity to adult resistance due to the timing of DEN injection is likely to be the consequence of age-dependent hepatocyte proliferation. Hepatocytes in perinatal rodent livers are actively proliferating, but after weaning the proliferation markedly slows down and is negligible within a few weeks (POST and HOFFMAN 1964). Proliferation of the target cells is known to play an important role in both the initiation and promotion stages of hepatocarcinogenesis (CRADDOCK 1976; HANIGAN *et al.* 1990). Interestingly, it has been reported that D2 mice are extremely sensitive to an exogenous promoter of hepatocarcinogenesis, phenobarbital, even if initiated with DEN at 5 weeks of age (DIWAN *et al.* 1986). Under these experimental conditions, D2 mice developed preneoplastic foci and liver tumors as frequently as did C3H mice, which are also responsive to phenobarbital (DIWAN *et al.* 1986; LEE *et al.* 1989a). The active proliferation of perinatal hepatocytes could act analogously to the promoting effect of phenobarbital in adult mice. Without such stimuli, the sensitivity loci may not express their phenotypes.

It is tempting to propose that the *Hcr* loci are responsible for the resistance of D2 mice to spontaneous hepatocarcinogenesis and DEN-induced hepatocarcinogenesis in adults. To test this hypothesis, we will construct a doubly congenic mouse strain in which the B6 alleles of *Hcr1* and *-2* are carried on a D2 genetic background, and we will determine the sensitivity of this congenic strain to various protocols for hepatocarcinogenesis. This congenic strain will also enable us to study the functions of the *Hcr* loci in terms of the stage of carcinogenesis during which they act and to attempt to identify some of the susceptibility loci carried by D2 mice using linkage analysis in animals from a backcross between the congenic and B6 mice.

We do not yet know whether the *Hcr* resistance genes act specifically on D2 mouse sensitivity genes or on a wider range of liver cancer susceptibility genes. For example, C3H mice are extremely sensitive to a broad variety of protocols for *in vivo* and *in vitro* hepatocarcinogenesis (GRASSO and HARDY 1975; DIWAN *et al.* 1986; DRINKWATER and GINSLER 1986; LEE *et al.* 1989a,b) and these sensitivities are determined by *Hcs* (*Hepatocarcinogen sensitivity*) loci (DRINKWATER and GINSLER 1986; BENNETT *et al.* 1993; GARIBOLDI *et al.* 1993). Again, using congenic mice, we would be able to test whether the *Hcr* genes are able to suppress the liver tumor (especially spontaneous liver tumor)-susceptible constitution of the C3H mouse. Our ultimate goal is to define the *Hcr* genes in molecular terms by cloning the genes based on their chromosomal positions and introducing them as transgenes into mice with genetic backgrounds that make them prone to develop liver cancer. Although the positional cloning of these genes requires substantial effort, it is essential to elucidate the molecular functions of the *Hcr* loci in order to gain insight into their abilities to prevent cancer development.

We thank Dr. WILLIAM F. DOVE for his critical reading of this manuscript, Dr. WILLIAM DIETRICH and Dr. JOHN TODD for providing information on the SSR markers, and Dr. ERIC S. LANDER for the Mapmaker/QTL program. We are also grateful for the excellent technical assistance of MARY L. WINKLER. This work was supported by grants CA-22484, CA-09135 and CA-07175 from the National Cancer Institute. L. M. B. was supported in part by a fellowship from the Procter and Gamble Corporation. R. A. C. is a Howard Hughes Medical Institute Predoctoral Fellow.

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Communicating editor: N. A. JENKINS