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C57BR/cdJ Hepatocarcinogen Susceptibility Genes Act Cell-Autonomously in C57BR/cdJ↔C57BL/6J Chimeras[1]

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ABSTRACT

Female C57BR/cdJ (BR) mice are unusually susceptible to spontaneous and chemically induced hepatocarcinogenesis relative to females of other inbred strains, in part because they are insensitive to the inhibitory effects of ovarian hormones on liver tumor development; BR males are intermediate among strains in their sensitivity. C57BL/6J (B6) male and female mice are relatively resistant among inbred strains. Linkage analysis of crosses between BR and resistant B6 mice identified two loci, on Chromosomes 17 and 1, that accounted for the high susceptibility of BR mice to hepatocarcinogenesis. To determine whether the increased susceptibility of BR relative to B6 mice is intrinsic to the target hepatocytes or is the result of local or systemic differences in milieu, we determined the strain of origin of tumors that arose in BR↔B6 aggregation chimeras. Chimeras were treated at 12 days of age with *N,N*-diethylnitrosamine, and individual tumors were dissected from 15 males at 32 weeks and from 7 females at 50 weeks of age. DNA was prepared from each tumor, and quantitative PCR assays were used to determine the strain of origin for each tumor. The overall contribution of each strain to non-neoplastic liver was determined using the PCR assay and through analysis of the relative amount of glucose phosphate isomerase activity associated with the BR and B6 electrophoretic variants; the median contribution of B6 cells to non-neoplastic liver was 50%. A majority (91%) of the 230 tumors analyzed from both sexes was derived from the BR donor, indicating that the net overall effect of BR susceptibility genes is cell autonomous.

INTRODUCTION

In humans and mice, the development of liver cancer is subject to environmental, genetic, and hormonal influences. In humans, the greatest risk factor for developing hepatocellular carcinoma is infection with hepatitis B or C virus, although other environmental factors, such as ingestion of aflatoxin B_1 or consumption of alcohol and tobacco products, also increase risk (1). Family and case control studies indicate that gene–environment interactions are important determinants of risk (2–4). Finally, throughout the world, the incidence of liver cancer in men is 2.6-fold higher than in women, indicating that sex hormones may influence the development of liver tumors (5, 6).

In mice, sex hormones are modifiers of liver cancer susceptibility. The results of gonadectomy experiments using several different inbred strains and F_1 hybrids indicate that testosterone promotes, and ovarian hormones suppress, liver tumor development (7–9). Although, in general, the rank orders of susceptibility among inbred strains for males and females are similar, BR[4] mice are a striking exception. Although male BR mice are intermediate among strains in their susceptibility, BR female mice are 20-fold more susceptible to liver tumor development than females of other inbred strains (10). In contrast to other strains, gonadectomy has little effect on liver tumor development in BR females (9). Two loci (*Hcf1* and *Hcf2* for *Hepatocarcinogenesis in females*), on Chromosomes 17 and 1, respectively, account for 85–90% of the increased susceptibility of BR mice relative to B6 mice (11). Intriguingly, males of the C3H and CBA inbred strains, which are particularly susceptible to both spontaneous and chemically induced liver tumors, also carry a major genetic determinant of susceptibility on Chromosome 1 (12).

Because the C3H strain is one of the most sensitive strains to liver tumor formation (10, 13), this strain has been studied extensively. Three studies indicate that the determinants of hepatocarcinogen susceptibility of the C3H strain act at the level of the target hepatocyte. The classic study of Condamine *et al.* (14) determined the origin of spontaneous liver tumors that arose in C3H↔B6 and C3H↔BALB/c chimeras by examining the differential histochemical staining properties of strain-specific variants of β-glucuronidase and by biochemical analyses of electrophoretic variants of additional enzymes. These authors found that the majority of tumors expressed the C3H-specific enzyme variants and concluded that the action of C3H susceptibility genes is cell autonomous. Two later studies determined the strain of origin of preneoplastic lesions in the livers of C3H↔B6 and C3H↔BALB/c chimeras that had been treated perinatally with DEN (15, 16). Serial sections of liver were stained with H&E to identify preneoplastic foci, and adjacent sections were stained immunohistochemically to reveal cells that expressed a C3H strain-specific antigen. These studies also concluded that the net effect of C3H susceptibility genes is cell autonomous.

There are several possible explanations for the increased susceptibility of BR mice relative to B6 mice and for the resistance of BR females to the suppression of hepatocarcinogenesis by ovarian hormones. Local effects would include differences in the target hepatocyte or its neighboring cells, whereas systemic differences would include factors such as differences in the levels of circulating ovarian hormones or in the indirect effects of ovarian hormones on the synthesis or secretion of products at other sites. We asked whether the factors that determine hepatocarcinogen susceptibility in BR mice act cell autonomously, within the target hepatocyte, or noncell autonomously by altering the local or systemic environment. We also asked whether the determinants act similarly in male and female BR mice. To answer these questions, we generated chimeras by aggregating embryos from B6 and BR mice and induced liver tumors by perinatal injection of DEN. We then analyzed DNA from liver tumors that arose in the chimeras to ascertain the strain of origin of the tumors.

MATERIALS AND METHODS

Animal Breeding. B6 and BR mice were bred in our facility from stocks purchased from The Jackson Laboratory (Bar Harbor, ME). B6-R26 (generation N10; Ref. 17) heterozygous mice were obtained from Dr. William F. Dove (McArdle Laboratory); they were bred subsequently by continued backcrossing to B6. Mice carrying the R26 insertion were identified by PCR genotyping at the closely linked marker *D6Mit36*. Mice were housed in plastic cages on corn cob bedding (bed-o'-cobs; Anderson Laboratory, Maumee, OH), fed

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[4] The abbreviations used are: BR, C57BR/cdJ; B6, C57BL/6J; C3H, C3H/HeJ; B6-R26, B6.129S7-*Gtrosa26*; DEN, *N,N*-diethylnitrosamine; IEF, isoelectric focusing; G6Pase, glucose-6-phosphatase; GPI, glucose phosphate isomerase; SSLP, simple-sequence length polymorphism.

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Mouse Breeder Diet (Harlan Teklad, Madison, WI), and acidified tap water *ad libitum*.

Production of Chimeras. Female B6, B6-R26 (N12), and BR mice between 4 and 6 weeks of age and weighing 14–18 grams were induced to superovulate and mated to males of the same strain. Chimeras were generated as described by Hogan *et al.* (18). Four to eight cell embryos were flushed from the oviducts of pregnant females, and the zona pellucida was removed by treatment with acid Tyrode's. Blastocysts were transferred to the uteri of pseudopregnant ICR females. Chimerism of the resulting animals was determined by observation of coat color.

Induction of Tumors. All mice were injected at 12 days of age with 0.05 μmol/gram body weight DEN (Eastman Kodak, Rochester, NY) dissolved in tricaprylin (0.01 ml/gram; Sigma, St. Louis, MO). Mice were sacrificed by CO_2 asphyxiation, at 30–34 weeks of age for males and 50–52 weeks for females. Individual tumors >1 mm in diameter on the surface of the liver were enumerated in all mice; in chimeric mice, individual tumors were carefully dissected and immediately frozen on dry ice. The remaining liver or composite sections of liver were collected and frozen on dry ice. Spleens or tail clips also were collected and stored frozen as a source of DNA.

DNA Extraction from Frozen Tissue. DNA was prepared by homogenizing tissue samples in 1.5-ml tubes with Teflon pestles in genomic lysis solution [20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 0.5% SDS, and 5 mM EDTA]. The starting volume for each sample was adjusted depending on the size of the tumor and ranged from 100 μl to 1 ml. Samples were digested overnight with 100 μg/ml proteinase K at 55°C, followed by incubation at 37°C with 100 μg/ml RNase A for 30–60 min. The samples were briefly chilled on ice, one-third volume of 6.25 M NH_4OAc (pH 6.5–7.0) was added, and then the samples were vortexed vigorously for 20 s. Samples were centrifuged at 12,000 × *g*; the supernatant was removed and precipitated with an equal volume of isopropanol or 2.5 volumes of 100% ethanol. DNA pellets were resuspended in 25–200 μl of 10 mM Tris, 1 mM EDTA, pH 8.0 (Tris-EDTA).

Assay for Determining the Origin of Tumors. The genotype of each tumor was determined using a quantitative PCR-based assay. Five SSLP markers (Ref. 19; *D1Mit1, D4Mit42, D15Mit5, D16Mit4,* and *D18Mit33*; Research Genetics, Huntsville, AL) were used to amplify DNA from individual tumors; primers for these markers produced polymorphic products from BR and B6 DNA and amplified DNA from both strains with approximately equal efficiency. Genomic DNA was quantified fluorometrically by Hoechst Dye staining. To quantify the amount of amplification product corresponding to each strain, the reverse primer of each SSLP primer pair was end labeled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, Arlington, IL). Between 25 and 250 ng of tumor DNA were amplified in a 20-μl reaction containing 0.13 nM forward and 0.13 nM radiolabeled reverse primers, 100 μM deoxynucleoside triphosphates, 1 × buffer (supplied as a 10 × stock with the Taq polymerase), and 0.35 units of Taq polymerase (Boehringer Mannheim, Indianapolis, IN). Amplifications were performed in a Perkin-Elmer GeneAmp PCR System 9600 (Norwalk, CT) using the following conditions: 94°C for 3 min followed by 25–30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 3 min at 72°C. Labeled products were separated by electrophoresis in denaturing polyacrylamide gels. The gels were dried, and the amount of product formed corresponding to each allele was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Determination of the Percentage of Chimerism of Normal Liver Tissue. A series of 10-μm-thick cryosections was cut from liver samples that had been stored at −80°C. Every seventh section was stained histochemically to reveal G6Pase activity, and the stained sections served as a template to identify non-neoplastic regions on unstained sections (20). Areas corresponding to normal liver (G6Pase-positive regions) were scraped with a scalpel blade and used as a source of DNA or protein. Material was scraped from several regions corresponding to different lobes of the liver and pooled. Samples for DNA extraction were placed in a tube containing Tris-EDTA plus 0.1% Triton X-100 and were immediately frozen on dry ice. Samples for protein homogenates were suspended in 35 mM NaCl, and 2.5 mM Na_2HPO_4 (pH 7.2) and immediately frozen. The percentage of contribution of each donor to the liver was estimated in two ways: (*a*) by determining the percentage of DNA from each donor using the same quantitative PCR assay used to type tumors; and (*b*) by evaluating the relative amounts of BR and B6-specific GPI activity. For the latter method, frozen samples were prepared for electrophoresis by three cycles of freezing and thawing to lyse the cells; protein homogenates were then electrophoresed on pH 3–10 IEF gels (NOVEX, San Diego, CA) to separate the GPI isoforms. The GPI bands were visualized using a colorimetric activity assay (21). A 0.4-cm-thick 2.5% Metaphor agarose (FMC, Rockland ME) gel slab containing 25 mM Tris-HCl, 2 mM citrate buffer (pH 8.0), 37.5 mM $MgCl_2$, 1 mg/ml fructose-6-phosphate, 3 mg/ml $NADT^+$, 6 mg/ml nitro blue tetrazolium, 10 μg/ml phenazine methosulfate, and 1 units/ml glucose-6-phosphate-dehydrogenase was placed on top of the IEF gel and incubated in the dark at 37°C for 30–60 min. Dark blue bands corresponding to areas of GPI activity developed in the agarose slab and, to a lesser extent, in the acrylamide gel.

Control samples containing various ratios of B6 and BR protein were generated by mixing liver homogenates prepared from B6 and BR male mice. The control livers were homogenized in 35 mM NaCl and 2.5 mM Na_2HPO_4 (pH 7.2), and the amount of total protein in the homogenates was determined using a Bio-Rad protein assay kit (Richmond, CA). The mixing controls were run concurrently with the samples from chimeras and used to estimate the relative ratios of GPI activity in the samples.

Sexing Chimeras. The sex of chimeric mice was determined by visual inspection of the genitalia at the time of weaning. Some of the animals used in this study could have resulted from male and female embryos that had been mixed together. To identify these mixed sex chimeras, DNA from the spleen, tail, or non-neoplastic sections of liver was tested using a PCR assay to detect the Y Chromosome-linked *Sry* locus (22). The primers used were SRY1, 5′-GAGAGCATGGAGGGCCAT-3′ and SRY2, 5′-CCACTCCTCTGTGACACT-3′. In addition, DNA was also analyzed using a PCR-RFLP assay that amplifies DNA from both the *Zfx* and *Zfy* loci (23). The primers used were ZFY1, 5′-ATAATCACATGGAGAGCCACAAGCT-3′ and ZFY2, 5′-GTCGCTTCTTTGGTATCTGAGAAAGT-3′. Products from the *Zfx* locus contain a *Hae*III restriction site, whereas the products of the *Zfy* loci do not. The PCR conditions for both the *Sry* and *Zfy/Zfx* assay were 94°C for 1.5 min, 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 1 min. The ZFY2 primer was end labeled with ^{32}P before adding it to the PCR reaction to allow quantitation. The PCR products were digested with *Hae*III and separated on a 2.5% Metaphor agarose gel. The gel was fixed in 5% acetic acid for 10 min, dried, and exposed to a PhosphorImager screen. The ratio of undigested to digested (*Zfy*:*Zfx*) product was determined using ImageQuant.

RESULTS

Induction of Liver Tumors. Liver tumors were induced by DEN in 15 male and 7 female B6↔BR chimeras. Because tumors develop more rapidly in males, males were sacrificed at 30–34 weeks of age, whereas females were sacrificed at 50–52 weeks. All tumors visible on the surface of the liver and >1 mm in diameter were counted and those tumors that were well separated from other tumors and large enough to be dissected free of contaminating surrounding tissue were excised and immediately frozen on dry ice. The number of tumors per liver observed in chimeric mice ranged from 8 to 122, with mean tumor multiplicities of 52 ± 30 for males and 56 ± 48 for females (Tables 1 and 2). These mean numbers of liver tumors were significantly greater than those observed for B6 males and females, which were 8.9 ± 10 and 3.9 ± 5.1, respectively ($P < 0.001$, Wilcoxon's rank-sum test). The mean tumor multiplicity for BR males was 48 ± 19, whereas for females, it was 52 ± 27, values that were not

Table 1 *Induction of liver tumors by DEN in B6↔BR chimeras and donor strains*

Twelve-day-old mice were injected i.p. with DEN (0.05 μmol/gram body weight). Males were sacrificed between 30 and 34 weeks of age and females at 50 and 52 weeks. Values are the mean number of tumors counted and the SD.

	Males		Females	
Strain	No. of mice	Mean tumor multiplicity (SD)	No. of mice	Mean tumor multiplicity (SD)
B6↔BR	15	52 (30)	7	56 (48)
BR	19	48 (19)	13	52 (27)
B6	21	8.9 (10.0)	27	3.9 (5.1)

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Table 2 *Analysis of tumors arising in B6↔BR chimeric mice*

Tumors were induced by i.p. injection with DEN (0.05 μmol/gram body weight) at 12 days of age. Males were sacrificed at 30–34 weeks and females at 50–52 weeks. Tumors were excised and frozen on dry ice.

	No. of tumors	No. analyzed	Origin of tumors[a]			Degree of liver chimerism
			BR	B6	Ind.[b]	% B6 (GPI)[c]
Males						
1[d]	23	9	7[e]	2[f]	0	>80
2[g]	9	8	3	3	2	>80
3[d]	56	15	9	3	3	>80
4[d]	63	16	1	5	10	>80
5	48	5	5	0	0	>80
6	84	18	18	0	0	>80[h]
7[d]	31	4	1	3	0	75
8[d,g]	64	17	17	0	0	50
9[g]	8	4	3	1	0	50
10[d]	95	12	11	1	0	<20
11[d,g]	38	7	7	0	0	<20
12[d]	97	24	24	0	0	<20
13[d,g]	71	20	20	0	0	<20
14	16	9	7	1	1	<20
15[g]	70	17	14	1	2	<20
Total [Mean ± SD]	773	185	147	20	18	[52 ± 25]
Females						
1	21	4	3	0	1	>80
2[g]	15	4	4	0	0	>80
3	60	12	9	1	2	50
4[d]	8	2	1	0	1	50
5	122	14	14	0	0	30[h]
6[g]	121	12	12	0	0	<20
7	45	19	19	0	0	ND[i]
Total [Mean ± SD]	392	67	62	1	4	[52 ± 29]

[a] The strain of origin of tumors was determined by analyzing DNA from tumors using a quantitative PCR assay. Three different SSLP markers were used to type each tumor DNA sample. Tumors were designated as derived from BR if >65% of the tumor DNA was BR, B6 if >65% of tumor DNA was B6, and indeterminate if DNA was not >65% derived from BR or B6.

[b] Indeterminate.

[c] Unless otherwise noted, the degree of chimerism in the liver was determined by comparing the relative activity of GPI-A with GPI-B.

[d] Mixed sex chimera as determined by the *Zfx:Zfy* ratio.

[e] Male by *Zfx:Zfy* ratio.

[f] Female by *Zfx:Zfy* ratio.

[g] R26 chimera.

[h] The degree of chimerism in the liver was determined by analyzing DNA from non-neoplastic areas using the quantitative PCR assay used to type tumors.

[i] Not determined.

significantly different from the corresponding groups of chimeric mice.

The PCR Assay Used to Genotype Tumors Is Quantitative. We used quantitative PCR to determine the strain of origin of tumors that arose in our chimeric mice. We randomly selected 11 different SSLP markers on various chromosomes and tested the markers by end labeling the reverse primer of each pair with ^{32}P and amplifying DNA from BR, B6, and B6BRF$_1$ animals. The PCR products were separated by electrophoresis in denaturing acrylamide gels, and the amount of product was determined. Five of the markers we tested (*D1Mit1, D4Mit42, D15Mit5, D16Mit4,* and *D18Mit33*) produced polymorphic products for BR and B6 alleles and amplified both alleles with approximately equal efficiency. Additionally, each marker in this set of five maps to a different chromosome, and none of these markers is associated with genomic regions that exhibit frequent loss of heterozygosity in mouse liver tumors (Refs. 24 and 25).[5] We next demonstrated that the PCR assay is linear by performing reconstruction experiments using each of the five primer pairs. B6 DNA was mixed with BR DNA to give a set of samples ranging from 100% B6 to 0% B6 (100% BR) by weight, and the PCR products were quantified. The PCR products from a mixing experiment after electrophoresis are shown in Fig. 1. The allelic ratio of product formed reflected the relative amount of input DNA and the assay was linear. The amount of DNA used in the PCR reactions to genotype tumors ranged from 25 to 250 ng; the assay was linear over this range. The regression lines generated using a set of mixing standards at 50 ng/reaction were used to determine the ratio of B6:BR DNA in tumors.

Criteria for Assigning the Origin of Tumors. DNA from each tumor analyzed was genotyped using at least three SSLP markers in the quantitative PCR assay. A representative gel used to genotype tumors is shown in Fig. 1. For each marker, a tumor was classified as BR if it contained >65% BR-derived DNA or B6 if it contained >65% B6-derived DNA. Tumors that did not contain >65% DNA from either strain were considered to be of indeterminate origin. A tumor was considered to be definitively classified if the assignment using all three markers was conclusive and in agreement. Some tumors could be definitively classified using one or two of the markers but were indeterminate using other markers. If the classification using three markers was not in agreement, the assays were repeated, additional markers were assayed, and the results for all markers were averaged. We chose 65% as the threshold for classifying tumors based on the assumption that tumors originating from one donor strain could arise in an animal predominantly derived from the other donor strain. In rat liver tumors, ≤40% of the cells (thus, ≤40% of the DNA) can be contributed by nonhepatocytes (26). Under the worst circumstance, 40% of the DNA isolated from a cleanly dissected tumor in a chimera could be derived from the strain that was not the precursor of the tumor. Although we were unable to classify some tumors, in most cases, the classification was clear cut (Fig. 2). For the majority of tumors analyzed in both sexes, >75% of the tumor DNA was derived from one strain or the other as determined by SSLP analysis.

The Extent of Chimerism in Normal Liver Tissue. We estimated the degree of chimerism in the livers of all 15 males and six of the

[5] T. M. Poole, T. A. Chiaverotti, and N. R. Drinkwater, unpublished observations.

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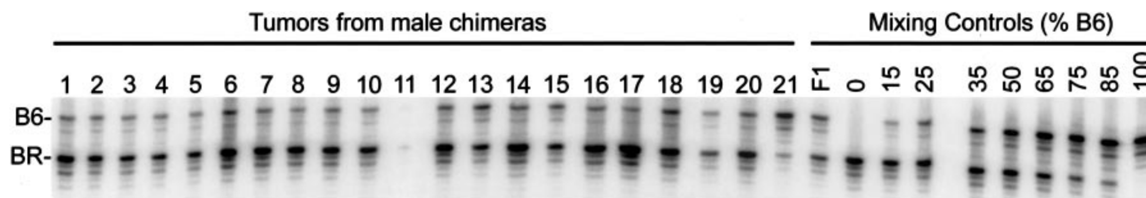

Fig. 1. Amplification of tumor DNA and mixing controls for marker *D18Mit33*. PCR products were separated by electrophoresis on denaturing polyacrylamide gels. The labeled products corresponding to the B6 (*top band*) and BR (*bottom band*) alleles were quantified.

seven females used in this study. Sections of liver were frozen on solid CO_2 at the time of sacrifice and stored at −80°C. A series of 10-μm-thick cryosections was cut from each liver, and every seventh section was stained histochemically to reveal areas of G6Pase activity. Hepatocellular preneoplastic and neoplastic foci are deficient in G6Pase activity (20). Slides containing unstained sections were aligned with the stained sections, and G6Pase-positive regions (normal tissue) were scraped using a scalpel blade. Scraped tissue was used as a source of DNA and protein homogenate. Hepatocytes account for only 65% of the nuclei in a rat or mouse liver but account for ~77–95% of the liver mass (27, 28); therefore, a greater proportion of total liver protein should be derived from hepatocytes relative to the amount of DNA.

We estimated the strain composition of the liver by evaluating the relative activity of GPI variants in protein homogenates from normal liver tissue. B6 mice express the GPI-1B isoform (isoelectric point = 8.7), whereas BR mice express the GPI-1A variant (isoelectric point = 8.4; Ref. 29). The two variants can be separated by electrophoresis on IEF gels, and the GPI bands were visualized using an activity stain. We conducted a mixing experiment to determine the limits of this method. Livers from B6 and BR mice were homogenized in 35 mM NaCl and 2.5 mM Na_2HPO_4 (pH 7.2), and protein homogenate from the B6 liver was mixed with BR liver homogenate to give a set of samples containing, by weight: (*a*) 100% B6 protein; (*b*) 75% B6; (*c*) 50% B6; (*d*) 25% B6; or (*e*) 0% B6 (100% BR). A total of 1.2 μg of protein from each control sample was electrophoresed on IEF gels and then stained for GPI activity. Bands corresponding to both isoforms were apparent in all lanes except for the 100% sample lanes. We classified the normal liver samples from chimeric mice by visual comparison with a set of mixing standards that had been run and stained concurrently. If we were unable to see a band at the position that corresponded to one of the isoforms, and if that sample contained enough total enzyme activity such that the band would have been visible if it was present, we determined that ≥80% of the hepatocytes were derived from the strain with the visible band. We confirmed our assignments by analyzing DNA using the quantitative PCR assay that was used to assign genotypes to tumors. The GPI results were in agreement with the DNA-based determinations (Kendall's test for independence, $P = 0.004$), and with one exception, the DNA- and protein-based determinations were within 15% of each other. The exception was a sample estimated to be 45% B6 using the DNA assay that did not produce a band corresponding to GPI-1B activity in the protein-based assay; it was classified as 80% BR.

Analysis of Tumors. A group of 185 tumors was dissected from the livers of 15 B6↔BR males; these samples represent ~24% of the observed tumors (Table 2). Of the 167 tumors that could be unambiguously assigned a genotype, 88% were derived from the BR donor. The degree of chimerism of the livers, in which tumors develop, must be considered to interpret fully these data. In this study, all three possible liver milieus were represented; in seven livers, the majority of hepatocytes was derived from the BR donor, two livers were approximately equal mixtures of B6 and BR hepatocytes, and six livers were predominately B6. In livers that were classified as predominantly BR, 83 of 89 tumors (93%) arose from BR cells; in livers that were comprised equally of BR and B6 hepatocytes, 20 of 21 (95%) tumors were of BR origin. Of the 75 tumors that arose in livers classified as predominantly B6, only 60 could be definitively classified; of these, 44 (73%) arose from the BR donor. Considering the group as a whole, the proportion of tumors arising from BR cells was significantly greater than the contribution of BR to non-neoplastic liver ($P < 0.001$, Wilcoxon's signed rank test).

Sixty-seven liver tumors from seven chimeric females were analyzed; these samples comprise ~17% of the total number of visible tumors (Table 2). Altogether, 62 of 67 (93%) tumors analyzed were BR in origin. Normal liver tissue in the seven females displayed a full range of chimerism. As in the males, the proportion of tumors derived from BR cells in chimeric females differed significantly from the fraction of normal liver derived from BR ($P < 0.03$). Combining the data from both sexes, 230 of 252 tumors could be definitively classified. Of these, 91% were of BR origin. Most important, in both sexes, there were animals with predominantly B6 livers, in which all of the tumors analyzed were from the BR donor.

Overall, 10% of the tumors in males were classified as indeterminate, and all of those arose in livers that were predominantly derived from one strain. We believe that many of the indeterminate tumors originated from the minority strain in those livers, and in one instance, we were able to demonstrate indirectly this origin. The liver of one mixed sex male was predominantly B6, and we were able to definitively classify two of nine tumors using SSLP markers. We used the *Zfy* locus to analyze DNA isolated from the spleen, normal liver, and tumors from that animal (Fig. 3). Spleen DNA was clearly positive for *Zfy,* whereas normal liver was negative for *Zfy*. Two tumors that were definitively classified as derived from the B6 donor using SSLP markers were negative for *Zfy,* whereas the seven tumors classified as indeterminate using SSLP markers were clearly *Zfy* positive and by inference of BR origin.

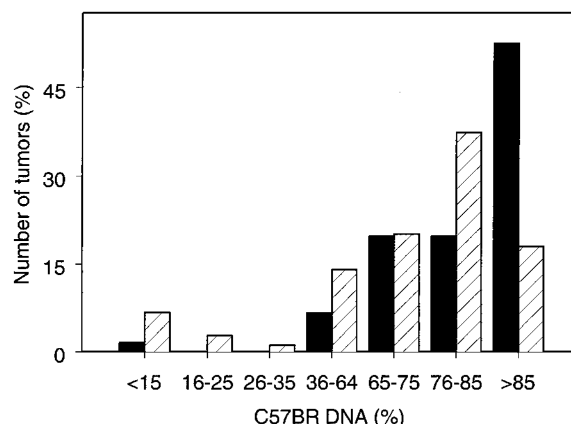

Fig. 2. Distribution of tumors as classified by percentage of BR DNA content. Tumors from males and females are represented by *striped* or *solid bars,* respectively.

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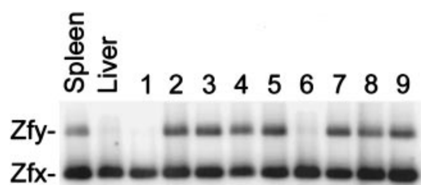

Fig. 3. Use of the *Zfy* locus to classify tumors from a mixed sex chimera. DNA isolated from spleen, liver, and liver tumors (*1–9*) was analyzed for *Zfx* and *Zfy*.

Eight of the chimeras in this study (six male and two female) were positive for the *R26* locus (Table 2). We intended to use R26 as a cell lineage marker to determine the origin of tumors, but we were unable to positively identify the R26 cell lineages in frozen sections using direct or indirect staining methods. We tested the possibility that the R26 insertion or linked loci had an effect on liver tumorigenesis and saw no significant difference in the origin of tumors that arose in B6↔BR chimeras (89% from BR among tumors definitively classified) compared with R26.B6↔BR mice (94% BR).

Molecular Determination of Sex. The sex of the mice in these experiments was determined by visual inspection at the time of weaning. One-half of the animals in this experiment would be expected to have resulted from male and female embryos that had been mixed together. In most cases where embryos of both sexes are mixed, the outcome is a phenotypic male. However, if no cells from the male migrate to the genital ridge, the resultant animal would be female. DNA samples from spleen or normal liver tissue from all of the mice used in this study were tested for the presence of the Y Chromosome-linked *Sry* locus to confirm retrospectively our assignments of gender. All phenotypic males were positive for the *Sry* locus. Although it was expected that all mice positive for the *Sry* locus would be phenotypic males, DNA from the spleen of one phenotypic female was positive for the *Sry* locus; the *Sry* locus was not detectable in DNA from normal liver or liver tumors in that mouse.

To determine how many mixed sex animals were used in this study, we used a quantitative PCR-RFLP assay to determine the *Zfy*:*Zfx* ratio. There are two Y-linked *Zfy* loci in mice and a single, X Chromosome-linked *Zfx* locus. All three loci can be amplified by PCR using the same oligonucleotide primers. The *Zfx* locus contains a *Hae*III restriction site that is absent in *Zfy* (23). We amplified the *Zfx* and *Zfy* loci from spleen and liver DNA using a ^{32}P end-labeled primer and then quantified the amount of *Zfx* and *Zfy* to determine whether an animal was of mixed sex. B6 and BR females had a *Zfy*:*Zfx* ratio ≤ 0.2, whereas males had a ratio ≥ 0.98. In this study, chimeras with a *Zfy*:*Zfx* ratio < 0.2 were classified as females; animals with a ratio > 0.98 were classified as male, and animals with a ratio between 0.2 and 0.98 were probably mixed sex. One phenotypic female had a *Zfy*:*Zfx* ratio of 0.66 and was also positive for *Sry*; this animal was the only mixed sex female in our study. Of the 15 phenotypic males in this experiment, 5 had a *Zfy*:*Zfx* ratio > 0.98, 4 had a ratio of 0.7–0.9, 5 others ranged from 0.2 to 0.7, and 1 male was not tested. Genotypically mixed sex males displayed the same bias in tumor development as single-sex chimeric males, with 87 and 89%, respectively, of the liver tumors derived from BR cells.

DISCUSSION

We used a quantitative PCR assay to determine the strain of origin of >250 liver tumors that arose in B6↔BR chimeras. Although, on average, the two strains contributed equally to non-neoplastic liver, 91% of the tumors that could be classified definitively arose from the BR donor. Our experiments demonstrate that the net effect of the genes that determine susceptibility to liver cancer in BR mice is cell autonomous in both males and females. If the increased susceptibilities to hepatocarcinogenesis of BR mice relative to B6 mice were attributable to systemic differences, hepatocytes from both donors in chimeras would experience the same systemic environment. Thus, the strain of origin of tumors that arose would reflect the contribution of each strain to the liver. On the other hand, if the difference between strains is intrinsic to the hepatocyte, the majority of tumors should arise from the more susceptible strain, regardless of the composition of the liver. Because hepatocytes derived from the B6 and BR strains experience the same hormonal environment in female chimeras, our results indicate that the mechanism by which ovarian hormones suppress liver tumor formation is hepatocyte specific and that BR hepatocytes are resistant to the suppressive effects even in a B6-like environment. We cannot formally exclude a model of increased BR susceptibility because of strain-specific microenvironments or from BR cell-specific juxtacrine signaling. However, we favor a cell autonomous interpretation because, even in livers primarily derived from the B6 donor, the majority of tumors arose from the BR donor, whereas very few B6-derived tumors arose in livers primarily derived from the more susceptible BR donor.

We were unable to classify definitively 22 tumors using quantitative PCR analysis. Fifteen of these tumors arose in livers of predominantly B6 origin but are likely to be of BR origin. In rat liver tumors, ~40% of the cells are nonhepatocytes (26). If mouse liver tumors contained similar proportions of nonparenchymal cells, we would not be able to classify tumors in which the neoplastic and non-neoplastic cells were derived from different strains. We were able to demonstrate in one mixed sex male that DNA samples we had at first classified as indeterminate using the SSLP-based PCR assay were positive for Y Chromosome-specific markers, and we inferred that the Y Chromosome-positive cells were from the BR donor.

The indeterminate tumors also could be polyclonal or collision tumors. However, data from studies using the C3H strain-specific antigen as a cell lineage marker suggest that ~100% of the preneoplastic lesions in C3H containing chimeras were monoclonal (15, 16). Although we were able to classify most of the tumors that arose in our study using the quantitative PCR assay, a cell lineage marker would have allowed us to determine the strain of origin of tumors, identify polyclonal tumors, and determine the patch size. The B6-R26 line has been successfully used as a cell lineage marker in the intestine (30), where it was useful for identifying polyclonal tumors (31). However, we were unable to use R26 as a lineage marker in the liver. Future analyses of chimeric livers may benefit from a recently described transgenic mouse line that uses the R26 promoter to drive high expression levels of human placental alkaline phosphatase (32) in the liver. The R26 insertion or a tightly linked locus has been reported to reduce the susceptibility of *Min* mice to intestinal and mammary tumors (33). If an R26-linked modifier locus acted cell autonomously in hepatocytes, the use of B6-R26 mice in this study could result in a bias for tumors derived from the BR donor. However, R26 (present in only 7 of the 22 chimeras we studied) did not significantly alter the strain distribution of tumors in chimeras compared with chimeras made using a pure B6 strain.

Male and female sex hormones have opposing effects on the development of liver tumors in mice (7–9). Testosterone acts noncell autonomously through the androgen receptor to promote liver tumor development (34), whereas in inbred strains other than BR, ovarian hormones inhibit tumor development (35, 36). Previous studies using chimeras also indicate that the hormonal environment of the animal rather than the genetic sex of the hepatocyte is the primary determinant of susceptibility to liver tumor development (15, 16). Thus, the gender of chimeric mice is best classified based on external genitalia, which reflects the hormonal environment. In this study, we first

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classified mice based on their external genitalia. We later determined that several phenotypic males and one phenotypic female resulted from mixing opposite-sex embryos and that in these animals, as in unisex mice, tumors were predominantly derived from the BR strain.

Two loci, *Hcf1* and *Hcf2,* account for most of the difference in susceptibility between the B6 and BR strains, and these loci act in both males and females to alter the growth or development of preneoplastic lesions (9, 11). Our results indicate that at least one of these loci acts within the hepatocyte, although both loci need not act in the same manner. BR and C3H mice share many traits related to the development of liver tumors. The BR and C3H hepatocarcinogen susceptibility loci *Hcf2* and *Hcs7,* respectively, map to the same interval on Chromosome 1, and a greater number of preneoplastic foci develop in the livers of BR and C3H males than develop in males of other strains at comparable times after treatment with DEN. Furthermore, both the BR and C3H susceptibility genes act cell autonomously. Our ultimate goal is to clone hepatocarcinogen susceptibility genes and study their activities at the molecular level. Knowing that some of the loci act cell autonomously will allow us to focus on genes expressed in the liver in our search for candidates.

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C57BR/cdJ Hepatocarcinogen Susceptibility Genes Act Cell-Autonomously in C57BR/cdJ <=>C57BL/6J Chimeras

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