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Mouse Strain Susceptibility to Diethylnitrosamine Induced Hepatocarcinogenesis Is Cell Autonomous Whereas Sex-susceptibility Is Due to the Micro-environment: Analysis with C3H↔BALB/c Sexually Chimeric Mice

Tetsuya Tsukamoto,[1,4] Ken-ichi Inada,[1] Hiroko Fukami,[1] Masami Yamamoto,[1] Harunari Tanaka,[1] Moriaki Kusakabe,[2] Colin E. Bishop[3] and Masae Tatematsu[1]

[1]*Laboratory of Pathology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464-8681,* [2]*Laboratory of Cell Biology, Tsukuba Life Science Center, RIKEN, Tsukuba, Ibaraki 305-0074 and* [3]*Departments of Obstetrics and Gynecology and Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, USA*

In man, liver cancer is on the increase, especially in males. Sex differences also exist in rodent models. To elucidate the mechanisms, chimeric mice were produced by amalgamation of early embryos from high and low hepatocarcinogen-susceptible strains, C3H and BALB/c. Tumor formation was initiated with 10 mg/kg of diethylnitrosamine at the ages of 7 and 14 days and mice were sacrificed at 30 and 45 weeks. The chimeras were classified into XY↔XY, XY↔XX, XX↔XY, and XX↔XX in terms of sex chromosomes by means of polymerase chain reaction-simple sequence length polymorphism analysis (SSLP) using Y chromosome-specific *Sry* primers in combination with the D3Mit21 marker. Liver lesions were analyzed histopathologically, by immunostaining using a C3H strain-specific antibody and by DNA *in situ* hybridization with the Y chromosome-specific digoxigenin-labeled Y353/B probe. Sex and strain genotyping by SSLP analysis matched histological observations, confirming the reliability of our system. The strain differences in liver tumor numbers of each strain type in XY↔XY and XX↔XX subtypes of C3H↔BALB/c chimeras were retained well ($P<0.0001$ and $P<0.001$, respectively), indicating a minimum influence of the C3H or BALB/c surrounding milieu on development of individual lesions. On the other hand, significant promotion of XX cell tumors was evident in phenotypically male sexually chimeric XY↔XX and XX↔XY chimeras for both C3H ($P<0.02$) and BALB/c ($P<0.01$) lesions compared to the XX↔XX case. The results suggest the presence of hormonal or micro-environmental factors specific for males, which are not caused cell-autonomously. Basic strain differences, however, are determined by intrinsic genetic factors rather than the strain-dependent micro-environment.

Key words: Chimera — Hepatocarcinogenesis — Strain susceptibility

In man, the occurrence of primary liver cancer has been on the increase for several years, changes in rates being more marked in males than in females.[1)] In Japan, 1996 death rates for intrahepatic neoplasia were 37.5 and 14.6/100 000 for males and females, respectively, according to the National Health and Welfare Statistics.[2)]

In rodent models, there also are sex differences in spontaneous and carcinogen-induced hepatocarcinogenesis, males being more susceptible than females.[3,4)] One fundamental question is whether the sex differences are cell-autonomous or caused by hormonal or micro-environmental factors. To address this issue, Kemp *et al.*[5)] utilized *Tfm* (testicular feminization) mutant mice and showed that liver tumor promotion by testosterone required a functional androgen receptor in the intact animal. Other researchers reported that castration of males reduced the incidence of carcinomas and adenomatous nodules in 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB)-treated[6)] and in *N,N*-diethylnitrosamine (DEN)-treated mice.[7)] The results with DEN-initiated and 2-acetylaminofluorene-treated rats appear similar.[8)] Furthermore, the incidences of liver tumors are increased in ovariectomized and DEN-treated female mice.[7)] However, it has not been possible to exclude a possible role of growth-promoting factors encoded by the Y chromosome or to compare XY and XX cells in the same environment. To elucidate the mechanism of sex differences, the chimeric mouse system, using hepatocarcinogen-susceptible and resistant strains, offers major advantages. Mintz *et al.*[9)] analyzed spontaneously developing liver tumors in C3H↔C57BL/6 chimeras utilizing the strain difference in malate dehydrogenase isozymes (*Mdh-1* locus). Lee *et al.*[10)] made analysis of microscopic liver tumors possible by using an antibody against the C3H strain-specific antigen (CSA).[11)] They clearly demonstrated that the principal mechanisms underlying strain differences in DEN-initiated hepatocarcinogenesis operate within the target cells rather than being milieu-dependent. Although they also analyzed karyotype sex by mating chimeras with C57BL/6 females, sex genotypes were still

[4] To whom correspondence should be addressed.
E-mail: ttsukamt@aichi-cc.pref.aichi.jp

mostly obscure. Therefore, overall analysis was conducted by mixing XY↔XY, XY↔XX, and XX↔XY subtypes of C3H/HeN↔C57BL/6N chimeras (strain-specifically described, i.e., left side is one strain and right one is the other) as phenotypically male animals.

In the present study, we induced liver tumors in DEN-treated C3H/HeN↔BALB/cA chimeras which were classified into XY↔XY, XY↔XX, XX↔XY, and XX↔XX subtypes using a newly developed strain-specific Sry simple sequence length polymorphism (SSLP) marker in combination with the strain-specific marker, D3Mit21, as well as the C3H strain-specific CSA antibody and the Y chromosome-specific Y353/B probe. We clarified that male-specific hormonal or micro-environmental factors are adequate for promotion of not only XY but also XX hepatocytes in sexually chimeric mice.

MATERIALS AND METHODS

Animals C3H/HeN (C3H) and ICR mice (originally purchased from Charles River Japan Inc., Kanagawa) and BALB/cA (BALB/c) mice (from CLEA Japan Inc., Tokyo) were maintained at the Animal Facility of Aichi Cancer Center Research Institute. They were housed in plastic cages with hardwood chips in an air-conditioned room with a 12 h light-12 h dark cycle and were given basal diet (Oriental NMF, Oriental Yeast Co., Tokyo) and water *ad libitum*.

Production of chimeric mice C3H↔BALB/c chimeric mice were produced by an aggregation procedure as described earlier.[11] Briefly, 8-cell stage embryos of C3H and BALB/c strains were collected by oviduct flushing with M2 medium (see ref. 12 for media components), and the zonae pellucidae were removed with acidic Tyrode solution. Each embryo was attached in M2 medium containing phytohemagglutinin and incubated in M16 medium at 5% CO_2 at 37°C. When aggregated embryos of each strain had reached the blastocyst stage, they were surgically transferred into the uterus of a pseudopregnant ICR foster mother. Chimeric animals showing the distinct chimeric coat color pattern were used in the analysis.

Experimental protocol Ten milligrams/kilogram body weight of DEN was injected intraperitoneally into 13 male and 8 female C3H mice, 13 male and 10 female BALB/c control mice, and 28 male and 8 female C3H↔BALB/c chimeric mice at 1 and 2 weeks after birth. The mice were sacrificed at 30 and 45 weeks old.

Determination of the strains and sexes of chimeric mice by SSLP analysis The procedure for SSLP analysis[13] was basically as described earlier.[14, 15] Genomic DNA of each chimeric mouse was isolated from frozen tissue with a QIAamp tissue kit (QIAGEN, Tokyo) or from paraffin sections with DEXPAT (TaKaRa, Ohtsu). To monitor the presence of DNA from both strains, D3Mit21F (5′-AAG CTC TAC AGC GGA AGC AC-3′) and D3Mit21R (5′-CTG GGG AGT TTC AGG TTC CT-3′) primers (Map Pairs, Research Genetics Inc., Huntsville, AL) were used. To determine the sex background for each of the strains, a 5′-primer (Sry7097U, 5′-GCA CAT TGT GGA GGA GAA CT-3′) and a 3′-primer (Sry7307D, 5′-CAC AGG CTG TGT CTC TTT AG-3′) for mouse *Sry*[16] were used. Strain differences could be distinguished with SSLP analysis due to the length of CA dinucleotide repeats between these two *Sry* primers. The 5 μl polymerase chain reaction (PCR) mixture consisted of 0.125 units of *Taq* DNA polymerase (TaKaRa), 1× buffer provided, 200 μM dNTP, 200 n*M* each of 5′- and 3′-primers, 50 n*M* [α-^{32}P]dCTP (NEG-513H, NEN Life Science Products, Boston, MA), and 1 μl of genomic DNA. PCR was performed as follows: 94°C 60 s×1 cycle: 94°C 45 s-55°C 30 s-72°C 60 s×40 cycles: 72°C 5 min. After PCR, 1 μl of 6× dye solution (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol, 20 m*M* EDTA) was added and 2 μl aliquots were loaded in 6% non-denaturing polyacrylamide gels (Long Ranger, FMC, BioProducts, Rockland, ME) and electrophoresed for 1.5 h at 80 W using a Poker Face II Nucleic Acid Sequencer (Hoefer Scientific Instruments, San Francisco, CA). X-ray film autoradiography was performed on dried gels with an intensifying screen for 16 h at −80°C.

Identification of C3H-derived cells by immunohistochemistry Livers were fixed in ice-cold 95% ethanol + 1% acetic acid overnight, dehydrated and embedded in paraffin. Serial sections for each animal were made for hematoxylin and eosin staining and CSA immunohistochemistry.[17] For the latter, deparaffinized and rehydrated sections on glass slides were irradiated at full power in a microwave oven (BioRad H2500, Richmond, CA) for 10 min at 95°C in 0.01 *M* citrate buffer, pH 6.0 to retrieve CSA antigenicity. Sections were then incubated with biotinylated mouse monoclonal anti-CSA antibody at a final concentration of 10 μg/ml followed by horseradish peroxidase-conjugated avidin (Zymed, Burlingame, CA) and visualized with 3,3′-diaminobenzidine hydrochloride (DOJIN, Kumamoto).

Identification of cells harboring Y chromosomes by *in situ* hybridization Serial or semiserial sections were made for Y chromosome-specific *in situ* hybridization. The Y353/B Y chromosome-specific probe[18] cloned into an *Eco*RI site of the pUC9 plasmid vector was used as a template for PCR based probe labeling. Briefly, pY353/B was digested with *Pvu*II (New England Biolabs, Inc., Beverly, MA), electrophoresed in a 0.8% agarose gel and purified with QIAEX II (QIAGEN). The purified insert (0.8 ng/ml) was used as a template for PCR using a PCR DIG Probe Synthesis Kit (Boehringer Mannheim, Tokyo) containing 2 n*M* each of 5′- (ATG ACC ATG ATT ACG CCA AGC) and 3′- (CCA GTC ACG ACG TTG TAA AAC) primers located at the polylinker site of pUC9 in the pres-

ence of digoxigenin-dUTP. PCR was performed following the manufacturer's instructions at an annealing temperature of 65°C. PCR products which migrated to 2 kb were purified with a QIAquick PCR Purification Kit (QIAGEN) and digested with *Msp*I (New England Biolabs) to 1.2 and 0.8 kb fragments. Since *Msp*I recognized CCGG in which digoxigenin-dUTP would not be incorporated within this sequence, it digested most of the PCR product. *In situ* hybridization was basically performed as described.[19] Sections were deparaffinized and rehydrated, incubated for 20 min in 0.2 *M* HCl at room temperature (RT), digested with 1 μg/ml proteinase K in 50 m*M* Tris-Cl, 5 m*M* EDTA, pH 7.5 at RT for 10 min, rinsed in 0.2% glycine in phosphate-buffered saline (PBS) at RT for 1 min, post-fixed with 4% paraformaldehyde for 15 min at RT, acetylated with 1/400 dilution of acetic anhydride in 0.1 *M* triethanolamine, pH 8.0, denatured in 70% formamide + 2× sodium chloride/sodium citrate (SSC) at 70°C for 5 min, chilled in 70% ethanol at −20°C for 2 min, transferred to 100% ethanol, and dried in air. Sections were washed in PBS between treatments. The DIG-labeled and *Msp*I-digested Y353/B probe was denatured at 100°C for 5 min, chilled on ice for 5 min, mixed in hybridization buffer (50% formamide, 10 m*M* Tris-Cl pH 7.5, 1 m*M* EDTA, 600 m*M* NaCl, 10 m*M* dithiothreitol (DTT), 1× Denhardt's solution, 0.25% sodium dodecyl sulfate (SDS), 10% Dextran sulfate, and 200 μg/ml *E. coli* tRNA) at 400 pg/μl, and applied to the sections with incubation at 50°C in a moist chamber containing 50% formamide + 2× SSC. After hybridization, slides were washed in 4× SSC, 50% formamide + 2× SSC, 2× SSC, and 0.2× SSC twice at 50°C for 15 min each. Signals were detected with a DIG Nucleic Acid Detection Kit (Cat. no. 1175 041, Boehringer Mannheim). Slides were soaked in 100 m*M* Tris-Cl, 150 m*M* NaCl, pH 7.5 (Buffer #1), and covered with the blocking solution for 30 min at room temperature. Then anti-DIG-AP diluted 1:500 was applied for 2 h at room temperature. Sections were washed twice in Buffer #1 for 15 min at the same temperature, soaked in 100 m*M* Tris-Cl, 100 m*M* NaCl, 50 m*M* $MgCl_2$, pH 9.5 (Buffer #3) for 2 min, and covered with color-substrate solution (500 μl of 337.5 μg/ml nitroblue tetrazolium salt and 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in Buffer #3) for 2–3 h at RT. Slides were washed in water and mounted with Crystal Mount (Biomeda Corp., Foster City, CA).

Pathological observation and statistical analysis A thousand CSA-positive (C3H) and negative (BALB/c) cells in normal regions of liver were counted and the chimerism ratio was calculated. The criteria for a focus and an adenoma were as described.[20] Briefly, the former was a lesion smaller than a normal lobule and the latter was a tumor bigger than a focus which maintained normal lobule structure with cellular atypia, but was not an invasive carcinoma. The numbers were counted for both strains and corrected for the ratio of chimerism. Invasive carcinomas were difficult to enumerate and were not included in this study. The areas of liver sections were measured using a color image processor (SPICCA, Nippon Avionics Co., Tokyo). The corrected values of foci + adenomas / cm^2 were statistically compared by use of the Mann-Whitney *U* test.[21]

RESULTS

Determination of sex genotypes of C3H↔BALB/c chimera Chimeras were produced by aggregation of two embryos from different strains. Since embryos were either male (XY for sex chromosomes) or female (XX), chimeras consisted of XY↔XY, XY↔XX, XX↔XY and XX↔XX combinations in terms of sex chromosomes. To analyze the incidence of tumor development in each strain and sex, it was necessary to determine the genotypes. The results of SSLP analysis performed to reveal the sex genotype for each strain in each chimera are summarized in Table I. Representative findings are shown in Fig. 1.

Table I. Sex Genotypes and Chimerism Ratio for C3H↔BALB/c Chimera Mice

Sex phenotype[a]	No. animals	Sex genotype[b]		Chimerism[c]	
		C3H	BALB/c	C3H	BALB/c
Male	12	XY	XY	0.51±0.17	0.50±0.17
Male	8	XY	XX	0.51±0.12	0.49±0.12
Male	8	XX	XY	0.64±0.09	0.36±0.09
Female	8	XX	XX	0.63±0.03	0.38±0.03

a) The male phenotype includes XY↔XY, XY↔XX and XX↔XY chimeras. Female chimera consists only of XX↔XX.
b) Sex genotypes were determined by SSLP analysis as shown in Fig. 1.
c) The chimerism ratios (mean±SD) were calculated after counting a thousand normal hepatocytes in CSA-stained sections. A chimera mouse with a proportion less than 0.20 of one strain was not included in the study.

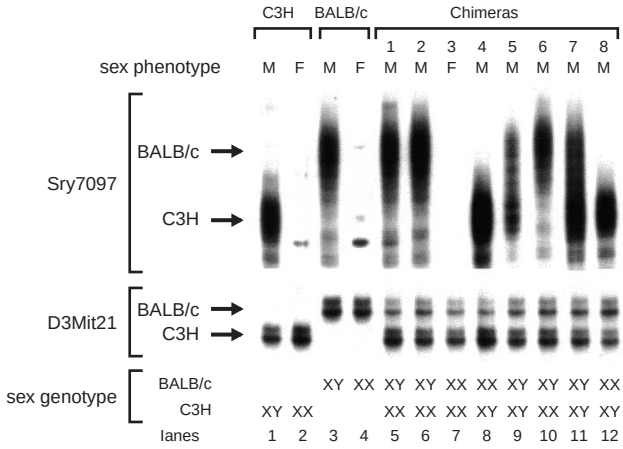

Fig. 1. Genetic determination of strain and sex background by PCR-SSLP analysis. D3Mit21 was used to monitor the presence of genomic DNA from both strains and Sry7097 (Sry7097U and Sry7307D primers) to determine the sex genotype. C3H and BALB/c show lower and higher bands in both sexes, respectively, with D3Mit21. Sry7097 is only visible in males. All chimeras were evaluated genotypically for strain and sex. Relatively equal D3Mit21 bands suggested comparable levels of the genomic DNAs from both strains. Chimeras 5 and 7 with both C3H and BALB/c bands were judged as XY↔XY. Chimeras 1, 2 and 6 had only the higher Sry band and were thus XY↔XX. Chimeras 4 and 8 possessed only the lower band and were judged as XX↔XY. Finally, the lack of any Sry bands for chimera 3 revealed it to be XX↔XX.

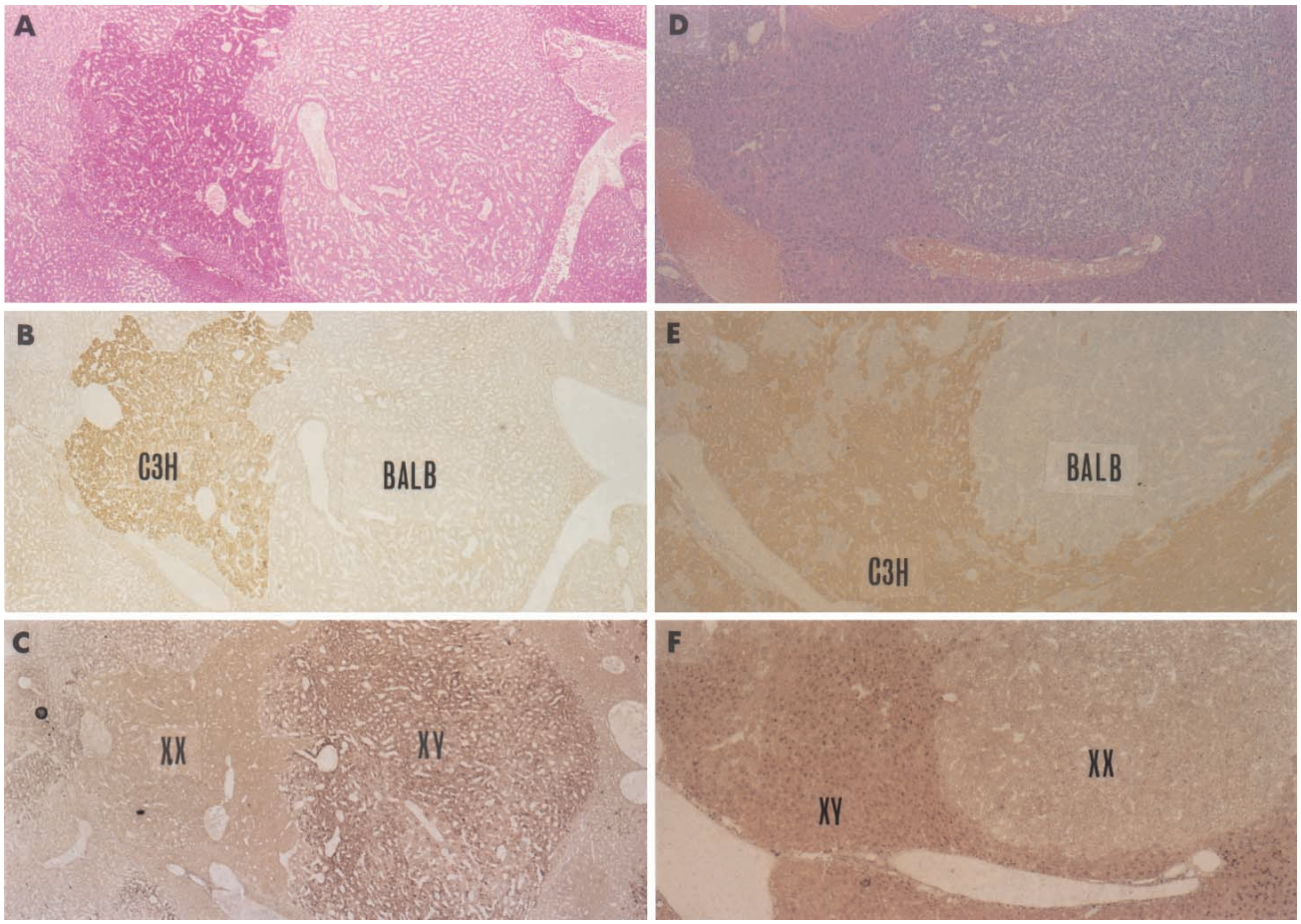

Fig. 2. Histopathological analysis of liver tumors in chimera 2 (A, B and C) and chimera 4 (D, E and F). Hematoxylin and eosin staining (A and D), CSA immunostaining (B and E), and Y353/B *in situ* hybridization (C and F). In chimera 2 (A, B and C), two tumors have collided with each other. That on the left is stained with CSA antibody (B) but was not hybridized with the Y353/B probe (C), whereas the opposite is the case for that on the right. The strain and sex combination was C3H (XX)↔BALB/c (XY). In chimera 4 (D, E and F), a tumor is located on the right-hand side, which is not stained with either CSA antibody or the Y353/B probe. The majority of surrounding cells are CSA and Y353/B-positive C3H (XY) cells. The strain and sex combination of chimera 4 was C3H (XY)↔BALB/c (XX).

Firstly, D3Mit21 was used to monitor the presence of genomic DNA from both strains, the C3H allele being smaller (Fig. 1, lanes 1 and 2) than that for BALB/c (Fig. 1, lanes 3 and 4) in both sexes. *Sry* primers, Sry7097U and Sry7307D, were designed to be located to amplify CA dinucleotide repeats. Male C3H and BALB/c exhibited smaller and larger bands, respectively, the number of CA repeats differing in these strains. In female mice, a faint background was visible in both strains (Fig. 1, lanes 2 and 4). This disappeared with hot start PCR using AmpliTaq Gold (Perkin-Elmer, Roche, Foster City, CA) (data not shown) indicating it represented a PCR artifact with conventional *Taq* polymerase. When this technique was applied to chimeras (Fig. 1, lanes 5–12), all showed roughly equal amounts of genomic DNA according to the intensity of their D3Mit21 bands. In chimeras #5 (lane 9) and #7 (lane 11), both C3H and BALB/c bands were visible and they were judged as XY↔XY. Chimeras #1 (lane 5), #2 (lane 6), #6 (lane 10) had only the larger Sry band and were therefore XY↔XX. Chimeras #4 (lane 8) and #8 (lane 12) possessed only the smaller band and were judged as XX↔XY. Finally, chimera #3 (lane 7) showed no Sry band, as expected for XX↔XX. The genotypically determined sexes coincided well with the phenotypically observed sexes. This novel Y chromosome-specific *Sry* primer set made it possible to determine strains as well as sexes not only in inbred strains, but also in chimeric mice.

CSA staining and Y353/B *in situ* hybridization analysis

Serial sections were stained with CSA antibody and the Y353/B probe along with hematoxylin and eosin. Representative results are shown in Fig. 2. All the tumors were either CSA- and Y353/B-positve or negative and monoclonal, consistent with previous results.[10] In Fig. 2, A–C, two adenomas are shown to have collided in chimera #2. CSA staining (Fig. 2B) revealed the left adenoma to be derived from C3H and the right one from BALB/c. Y353/B *in situ* hybridization (Fig. 2C) revealed only the right adenoma to be from XY. Thus, the C3H↔BALB/c chi-

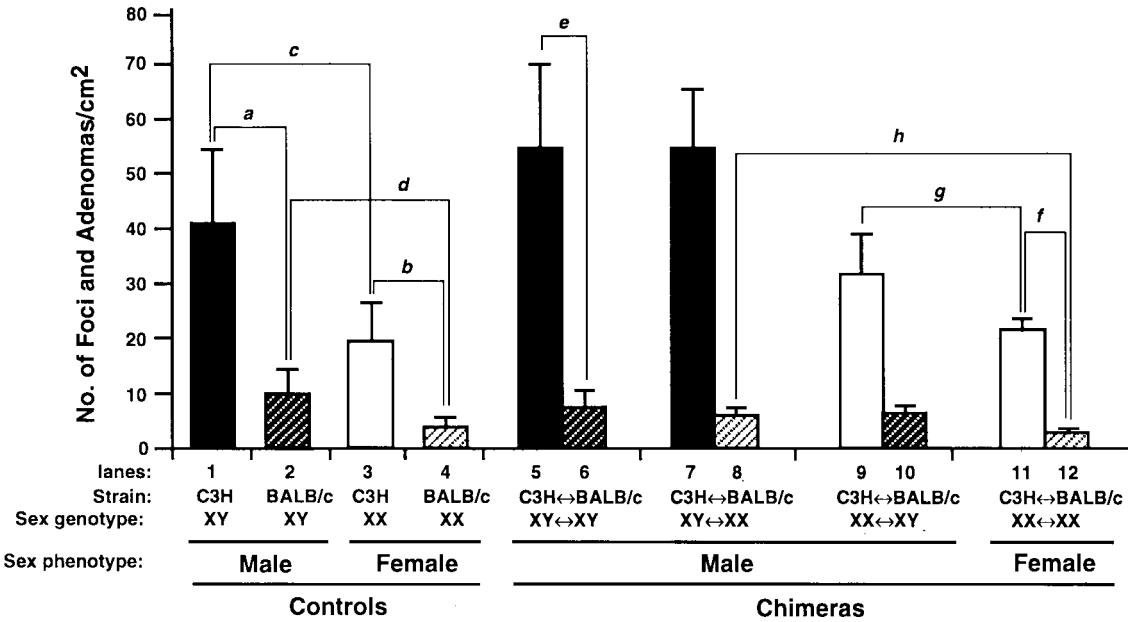

Fig. 3. Numbers of liver foci+adenomas/cm^2 (mean±SD). In the control strains (lanes 1–4), the values for male and female C3H (lanes 1 and 3) were 41.6±13.2 and 19.2±7.4, respectively. In BALB/c, they were lower in both males (lane 2, 10.1±4.1) and females (lane 4, 3.9±2.1). Significant strain differences were noted in both males (comparison *a*, $P<0.0001$) and females (*b*, $P<0.0001$). Comparing numbers by sex in each strain, males were significantly more susceptible in C3H (*c*, $P<0.0005$) and BALB/c (*d*, $P<0.0005$) cases. C3H↔BALB/c chimeras (lanes 5–12) were analyzed and values were corrected for the ratio of chimerism. In C3H (XY)↔BALB/c (XY) males (lanes 5 and 6), the values for C3H (lane 5) and BALB/c (lane 6) tumors were 54.5±15.3 and 8.2±2.7, respectively, and the strain difference was maintained (*e*, $P<0.0001$). The same was the case in C3H (XX)↔BALB/c (XX) female chimeras (*f*, $P<0.001$), where the numbers of C3H (lane 11) and BALB/c (lane 12) tumors were 20.9±3.5 and 3.6±0.7, respectively. For the analysis of the male hormonal environment, XY↔XX and XX↔XY chimeras (lanes 7–10) were compared with XX↔XX (lanes 11 and 12). The number of C3H (XX) tumors (lane 9) in C3H (XX)↔BALB/c (XY) chimeras and that of C3H (XX) tumors (lane 11) in C3H (XX)↔BALB/c (XX) animals were 32.1±7.0 and 20.9±3.5, respectively. C3H (XX) cells were more susceptible in the male environment (*g*, $P<0.01$). Similarly, the value for BALB/c (XX) tumors (lane 8) in C3H (XY)↔BALB/c (XX) chimeras and that of BALB/c (XX) tumors (lane 12) in C3H (XX)↔BALB/c (XX) animals were 6.0±2.0 and 3.6±0.7, respectively. BALB/c (XX) cells were also more susceptible in the male environment (*h*, $P<0.02$).

mera #2 could be judged as XX↔XY histologically. In the case of chimera #4 (Fig. 2, D–F), an adenoma is seen on the right side (Fig. 2D) which was not stained with CSA antibody and therefore judged to be derived from BALB/c (Fig. 2E). *In situ* hybridization revealed that this adenoma did not possess a Y chromosome, thus indicating XX (Fig. 2F). Therefore, the C3H↔BALB/c chimera #4 was elucidated to be XY↔XX. The histological results coincided well with those of SSLP analysis (Fig. 1).

Histopathological analysis Mice were sacrificed at 30 weeks old and numbers of foci + adenomas per cm^2 were calculated (Fig. 3, lanes 1–4). The values for male and female C3H mice (lane 1 and 3) were 41.6±13.2 and 19.2±7.4 (mean±SD), respectively. In BALB/c, values were lower in both males and females (Fig. 3, lanes 2 and 4) at 10.1±4.1 and 3.9±2.1, respectively. Significant strain differences between C3H and BALB/c were evident for both sexes (Fig. 3, comparisons *a* and *b*; male, $P<0.0001$ and female, $P<0.0005$). Comparing incidences in males and females in each strain, males were significantly more susceptible in C3H (Fig. 3, *c*, $P<0.002$) and in BALB/c (*d*, $P<0.001$). C3H in terms of strain, and males in terms of sex were more susceptible to DEN-induced hepatocarcinogenesis.

To analyze whether the susceptibility to tumor induction by DEN was cell autonomous or influenced by the hormonal or micro-environment, we used C3H↔BALB/c chimeras (Fig. 3, lanes 5–12). In C3H (XY)↔BALB/c (XY) male chimeras, it was possible to compare strain differences in the same male environment irrespective of the strain-specific environment. The number of C3H tumors (Fig. 3, lane 5) was 54.5±15.3, whereas that of BALB/c tumors (Fig. 3, lane 6) was 8.2±2.7. The strain difference (Fig. 3, comparison *e*, $P<0.0001$) was thus rather slightly widened in chimeras. In C3H (XX)↔BALB/c (XX) female chimeras, the numbers of C3H (Fig. 3, lane 11) and BALB/c (Fig. 3, lane 12) were 20.9±3.5 and 3.6±0.7, respectively. The strain difference was also maintained (Fig. 3, comparison *f*, $P<0.001$).

We next compared the alteration of incidence of genetically similar cells in different hormonal environments. The numbers of C3H (XX) tumors in C3H (XX)↔BALB/c (XY) chimeras (Fig. 3, lane 9) and in C3H (XX)↔BALB/c (XX) animals (Fig. 3, lane 11) were 32.1±7.0 and 20.9±3.5, respectively. C3H (XX) cells were more susceptible in the male environment with statistical significance (Fig. 3, comparison *g*, $P<0.01$). The numbers of BALB/c (XX) tumors in C3H (XY)↔BALB/c (XX) chimeras (Fig. 3, lane 8) and C3H (XX)↔BALB/c (XX) animals (Fig. 3, lane 12) were 6.0±2.0 and 3.6±0.7, respectively. Thus, BALB/c (XX) cells also became more susceptible in the male environment (Fig. 3, comparison *h*, $P<0.02$). The results suggest that tumor progression of genetically XX cells was promoted in the male environment.

DISCUSSION

The present study using the C3H↔BALB/c chimeric mice system with defined sexual mosaicism provided evidence that mouse strain susceptibility to hepatocarcinogenesis is derived cell-autonomously and that the sex susceptibility, in contrast, is due to the hormonal environment.

Sexual mosaicism is known to occur frequently in phenotypically male chimeric mice.[9] For the C3H↔BALB/c chimeras used in this study, combinations in terms of sex chromosomes were XY↔XY, XY↔XX, XX↔XY and XX↔XX (note that the left side of the arrow is for C3H and the right one is for BALB/c). Sex chromosome analysis in chimeric mice has previously been achieved by karyotype preparation[9, 22] or mating and evaluation of offspring.[10] However, these methods are time-consuming and do not reveal the karyotype of sex chromosomes in a strain-specific manner. Therefore, we searched for a strain-specific, Y chromosome-specific microsatellite marker on the *Sry* gene which has several dinucleotide repeats and found SSLP primers. For analysis of strains and sexes, the presence of DNA from each strain could be semiquantitatively confirmed with an autosomal microsatellite marker such as D3Mit21. The corresponding bands for each strain in *Sry* PCR products was compared (Fig. 1). With this method, analysis of chimerism including strains and sexes can be quickly completed even with biopsy samples taken, for example, from tails. Since C57BL/6 shows an identical pattern to BALB/c (manuscript in preparation), this marker can be used for C3H↔C57BL/6 chimeras as well.

For the analysis of tumorigenesis in chimeric animals, an ideal histological marker should fulfill seven criteria[11, 14]: (i) be cell-localized, i.e. not secreted extracellularly; (ii) be cell autonomous, i.e. not transferred between cells or affect other cells; (iii) be stable both within the first marked cells and all of their mitotic progeny; (iv) be ubiquitous throughout development; (v) be easy to detect; (vi) be developmentally neutral, not causing cell selection or influencing developmental processes; and (vii) be stable during tumorigenesis.

CSA has been shown to be identical to peptide-binding protein 74 (PBP74), a member of the stress-70 protein family[23] localized in mitochondria,[24, 25] whose gene locus is on chromosome 18.[26] The immunogenicity of CSA derives from the substitution of 2 amino acids in the substrate recognition domain of this protein.[25] Although PBP74/CSA has been reported to be constitutively expressed,[24, 25, 27] immunohistochemical detection of CSA may be affected by quantitative or qualitative changes of PBP74/CSA expression. Changes in the level of PBP74/CSA expression in tumor tissues have not yet been thoroughly examined, though CSA has been used as a stably expressed marker for tumors of the liver,[10] forestomach,[14]

glandular stomach,[22] colon,[15] and urinary bladder.[28] To re-confirm the stability of CSA expression in liver tumors, another marker was sought to provide complementary results. Since CSA antibody was designed to discriminate strains, sexual chimerism was utilized to distinguish the two strains, albeit limited to XY↔XX and XX↔XY chimeras. Thus, the Y353/B Y chromosome-specific marker[18] was chosen, because 30 copies of the cognate and 250 copies of related sequences exist along the length of the Y chromosome.[29] This chromosomal marker has been used as a hemizygous marker for liver sections[30] and for transplanted hepatocytes[31] with strong reproducibility. The Y chromosome-specific marker can visualize XY cells directly even when the chimerism ratio is extreme so that *Sry* SSLP analysis is impossible. Targets for DNA *in situ* hybridization are generally thought to be stable. A transgene with a 1000 tandem repeat of β-globin in transgenic mice[32] was readily detected by DNA *in situ* hybridization in histological sections. It has been proven to fulfill many of the requirements of an ideal genetic cell marker and widely used for lineage studies with mouse chimeras.[33] Patek *et al.*[34] used a Y chromosome-specific pM34-2/0.6t probe for the analysis of sex chimerism of XX↔XY chimeras. As shown above, DNA *in situ* hybridization is a powerful tool for analysis of XY↔XX and XX↔XY chimeras. In this study, Y353/B probe visualized XY cells successfully in our chimeras. CSA staining and Y353/B DNA *in situ* hybridization patterns (Fig. 2) matched with SSLP data using D3Mit21 and *Sry* primers (Fig. 1). Thus, CSA expression was considered to be as stable as the Y chromosome in liver tumors.

The C3H strain is very susceptible to DEN-induced hepatocarcinogenesis.[4, 27, 35] Strain differences in both pure male (XY↔XY) and female (XX↔XX) chimeras were maintained (Fig. 3, comparisons *e* and *f*) with statistical significance ($P<0.0001$ and $P<0.001$, respectively) in the present study. The strain difference in liver tumor development in each strain in XY↔XY and XX↔XX chimeras was retained, indicating no enhancing effect of the C3H background on BALB/c cells or on the inhibitory effect of the BALB/c background. Thus, the susceptibility to induction of liver tumors in the C3H strain can be considered cell-autonomous, in accordance with previous results.[10] The widening of differences could actually have been caused by the rapid development of hepatocarcinogen-sensitive C3H liver cells[36] compared to the rather resistant BALB/c hepatocytes. Expansion of C3H tumors could have hindered BALB/c tumor growth as well.

In sexual chimeras, the effects of the male hormonal environment were examined by utilizing lesions comprised of XX cells. Comparing C3H (XX) cells in male and female environments (Fig. 3, lane 9 vs. 11), the incidence was increased in males (Fig. 3, comparison *g*) with statistical significance ($P<0.01$). The same analysis of BALB/c (XX) cells (Fig. 3, lane 8 vs. 12) also revealed a significant increase in males (Fig. 3, comparison *h*, $P<0.02$). Thus, the sex difference in susceptibility is determined by the male hormonal environment, implicating testosterone as a major factor. Kemp and Drinkwater[37] demonstrated that the testosterone-androgen receptor axis is important for liver tumor promotion by taking advantage of *Tfm* mutant mice lacking functional androgen receptors. Although plasma testosterone levels did not correlate with strain-specific susceptibility,[37] castration reduced the tumor development in males and ovariectomy yielded higher incidences of liver tumors in females.[6–8]

Finally, we believe that the new *Sry* SSLP marker, utilized in combination with the CSA antibody and Y353/B probe, opens up new avenues not only to studies of hepatocarcinogenesis but also to any research into sex differences in chimeric mice. It should thus help clarify racial and sexual differences in human cancer development.

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