Inheritance of Resistance to Promotion of Preneoplastic Liver Lesions in Copenhagen Rats

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Previously, we have shown that Copenhagen (Cop) rats are highly resistant to the induction of putative preneoplastic, glutathione *S*-transferase 7-7- (GST 7-7) positive liver lesions following treatment with a modified resistant hepatocyte (RH) protocol. The objective of this study was to determine if resistance is inherited in a dominant or recessive manner and to derive an estimate of the number of genetic loci involved. We crossed male and female Cop rats with F344 rats to produce F1 offspring. Backcross rats were generated using female F1 rats and either Cop or F344 males, resulting in B1c and B1f generations, respectively. The male rats from all these crosses were initiated with diethylnitrosamine (200 mg/kg) at 7 to 8 weeks of age and were promoted 3 weeks later with the RH protocol (2-acetylaminofluorene and a two-thirds partial hepatectomy). The rats were sacrificed 3 weeks after the partial hepatectomy and their livers were sectioned and stained for GST 7-7-positive lesions. The susceptibility of F1 rats was in between Cop and F344 rats, having 21.7% ± 2.0% (mean ± SEM) of their liver volume occupied by lesions versus 4.2% ± 0.8% for Cop and 53.0% ± 5.8% for F344 rats. As expected, B1c rats had a volume of liver occupied by lesions that was in between the F1 and Cop rats at 13.5% ± 1.6%. Surprisingly, B1f rats were similar to B1c rats in their resistance (9.1% ± 2.1%). These results point to a complex, polygenic inheritance pattern that can be explained by a minimum of four loci, one of which shows recessive epistasis.

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Hepatocellular carcinoma (HCC) is one of the most frequent cancers worldwide and is most often associated with exposure to environmental factors such as aflatoxin B1, hepatitis viruses B and C, and alcohol consumption (1). Clearly, not everyone exposed to these agents develops HCC, and genetic factors are likely to be involved. The rat model of hepatocarcinogenesis is well established and lends itself to the investigation of inherited susceptibility and resistance since different strains of rat have different levels of susceptibility (2–4). Copenhagen (Cop) rats are resistant to HCC induced by long-term feeding of 2-acetylaminofluorene (2-AAF) (5). We have shown that Cop rats are highly resistant to the formation of preneoplastic liver lesions induced by diethylnitrosamine (DEN) and promoted using the modified resistant hepatocyte (RH) protocol (2). Compared to susceptible F344 rats, Cop rats had ~10-fold less of their liver volumes occupied by glutathione *S*-transferase 7-7- (GST 7-7) positive lesions 3 weeks following treatment with the RH protocol (6).

Proliferation and migration of oval cells occurs in F344 rat livers following treatment with the RH protocol (7). In contrast to oval cells in F344 rats, these cells fail to migrate in Cop rats during promotion with the RH protocol and they remain localized to the periportal areas (6). The migration of oval cells is associated with the production of several growth factors, including hepatocyte growth factor, transforming growth factor α, acidic fibroblast growth factor, and stem cell factor (8, 9), which may contribute to the growth of preneoplastic lesions. Thus, the less extensive oval cell migration that we observed in Cop rats may play a role in their resistance to the growth of GST 7-7-positive lesions. The DRH strain of rat is also resistant to liver carcinogenesis and, like the Cop rat, has less oval cell response than susceptible Donryu rats (10).

We have shown that the apoptotic and proliferative indices of lesions are not different between Cop and F344 strains, but lesions in Cop rats clearly do not increase in size at the same rate as those in F344 rats (6). There is a high degree of remodeling, however, in Cop lesions. Remodeling is the process whereby lesions lose expression of marker enzymes such as GST 7-7 and become more like normal hepatocytes (11, 12). Thus, Cop lesions may grow at a

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similar rate to F344 lesions, but then begin to remodel into normal appearing liver by day 14 of the RH protocol. Like Cop rats, preneoplastic liver lesions in resistant Brown Norway (BN) rats also have been reported to have a high rate of remodeling (3). Although a high rate of remodeling is a possible mechanism for resistance, it is poorly understood, and may occur spontaneously when lesions stop growing. Thus, remodeling could be a consequence rather than a cause of resistance.

Studies of the BN and DRH rats have shown that resistance is transmitted in a dominant manner in both strains (3, 10). In the BN rat, five potential loci have been associated with resistance (13). Two clusters of loci have been associated with resistance in DRH rats (14). Like Cop rats (6), both these strains appear to be resistant to the promotion stage of liver carcinogenesis, but not to initiation (3, 10). The similarities between these rat strains suggest that they may share common resistance mechanisms. Since the pattern of inheritance of resistance in Cop rats is unknown, the objective of this study was to determine if resistance is inherited in a dominant or recessive manner, and to derive an estimate of the number of loci involved.

Materials and Methods

Chemicals. DEN (Eastman Kodak Co., Rochester, NY) was >98% pure by gas chromatography. Normal swine serum and swine anti-rabbit biotinylated antibody were from DAKO (Mississauga, Ontario, Canada). 2-AAF and X-Gal were from Sigma Chemical Co. (St. Louis, MO). The rabbit anti-rat GST 7-7 antibody was a gift from Dr. Tom Rushmore (15) and the streptavidin-β-galactosidase conjugate was purchased from Boehringer Mannheim (now Roche Biochemical, Dorval, Quebec, Canada).

Animals. Cop and F344 rats from Harlan Sprague-Dawley (Indianapolis, IN) were bred to produce F1 rats with either Cop or F344 dams. Female F1s were crossed with either Cop or F344 males to produce backcross rats designated B1c and B1f, respectively. Food (Harlan Teklad, 6% fat, Madison, WI) and acidified water (pH 2.8) were provided *ad libitum* and a 12:12-hr light:dark cycle was maintained automatically. The protocols used were approved by the animal care committee of the Ontario Cancer Institute.

Animal Treatments. Seventeen F1s with Cop dams, 18 F1s with F344 dams, 19 B1c, 23 B1f, 9 Cop, and 10 F344 rats, all males, were administered a single intraperitoneal dose of 200 mg/kg DEN dissolved in 0.9% NaCl solution at 7 to 8 weeks of age. All rats were then treated using a modified RH protocol (16). Briefly, 18 days after DEN, three daily gavages of 20 mg/kg 2-AAF in DMSO and corn oil (1:29, v/v) were given followed by a two-thirds partial hepatectomy (PH). A fourth dose of 2-AAF (5 mg/kg) was given 4 days after PH. All rats were sacrificed 21 days after PH and liver samples were fixed in 5% acetic acid in methanol.

Immunohistochemical Staining. After fixing for 3 to 4 hr, tissues were processed, embedded in paraffin wax, and 2-μm sections were prepared on microscope slides. Immunohistochemical staining was carried out for GST 7-7 using X-Gal as a chromogen, as described by Stinchcombe *et al.* (17). Briefly, following deparaffinization and rehydration, sections were blocked with 10% normal swine serum for 10 min and then rabbit anti-GST 7-7 antibody was applied (1:2000) overnight at 4°C. The following were then applied to the sections at room temperature, washing with PBS between steps: biotinylated swine anti-rabbit antibody at 1:500 for 2 hr, streptavidin-β-galactosidase complex at 1:200 for 2 hr, and X-Gal substrate for 1 hr. All dilutions were made in 1% normal swine serum in PBS. Sections were finally counterstained with H+E (hematoxylin and eosin) and mounted.

Analysis of Stained Sections. The areas of GST 7-7-positive lesions in liver sections were measured as described previously (2) using quantitative morphometry (Bioquant IV, Zeiss instruments, Wetzlar, Germany) and were converted to the percentage of liver volume occupied by lesions and the number of lesions per liver using the method of Enzmann *et al.* (18) as we have described previously (6). Only lesions with radii greater than 35 μm were included in the analysis (3, 19). Previously, we have found that large lesions in F344 rats coalesce as they grow, making the number and size of individual lesions inaccurate measures of promotion (6). For this reason, we have not used the number of lesions or average lesion size as phenotypic parameters in this study. Lesions that showed evidence of remodeling (patchy or weak GST 7-7 staining and/or indistinct borders) were included in the analysis. Oval cells were identified by their characteristic ovoid nucleus and scant cytoplasm (7).

Statistical Tests. Results were analyzed using a one-way analysis of variance (ANOVA) and a Tukey post test was used to compare one group of animals with another.

Results

Percent Liver Volume Occupied by GST 7-7-Positive Lesions. Male rats from the two parental strains (Cop and F344), F1's, and backcrosses to male Cop (B1c) and male F344 (B1f) were treated with DEN and promoted using an RH protocol. They were sacrificed 3 weeks later and the percentage of liver volume occupied by GST 7-7-positive lesions was measured in individual rats. Because each male F1 rat has either a Cop or F344 dam, differences in susceptibility between these two F1 groups would provide evidence of sex linkage or maternal influence on the inheritance of resistance. Since F1's with either Cop or F344 dams were not significantly different from each other (18.7% ± 2.6% vs 24.0% ± 2.9%, respectively) there is no evidence for sex linkage or maternal influence, and the two F1 groups were combined for all subsequent analyses. Similarly, F1 females that had either Cop or F344 dams produced backcross offspring that did not differ in resistance (data not shown) and so these groups (B1c and B1f)

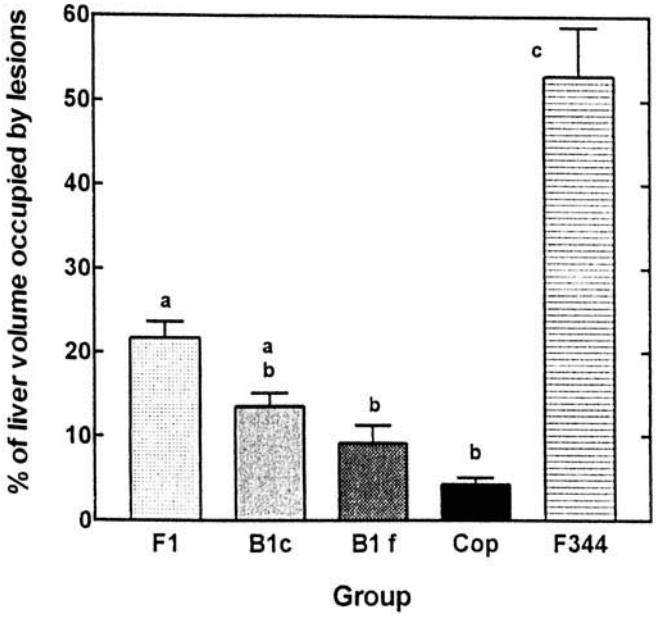

Figure 1. Percentage of liver volume occupied by GST 7-7-positive lesions in rats treated with a modified RH protocol and sacrificed 21 days after PH. Error bars are the SEM of each group. Groups sharing the same letter are not significantly different ($P < 0.05$).

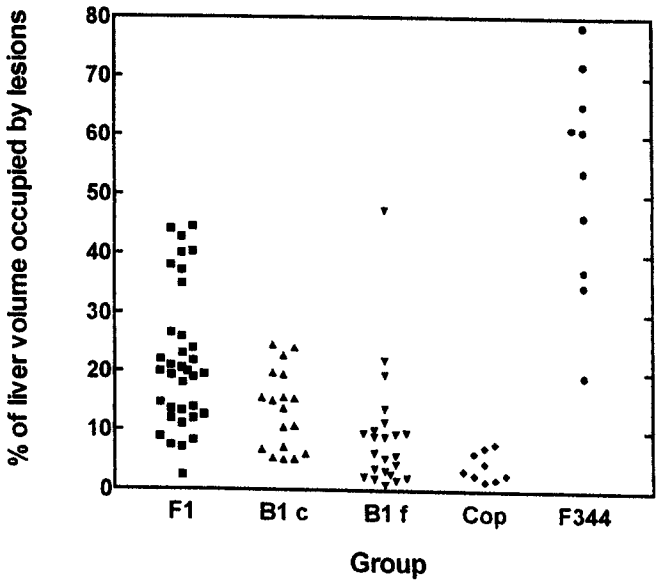

Figure 2. Scatter-plot of data in Figure 1. Each point represents an individual rat.

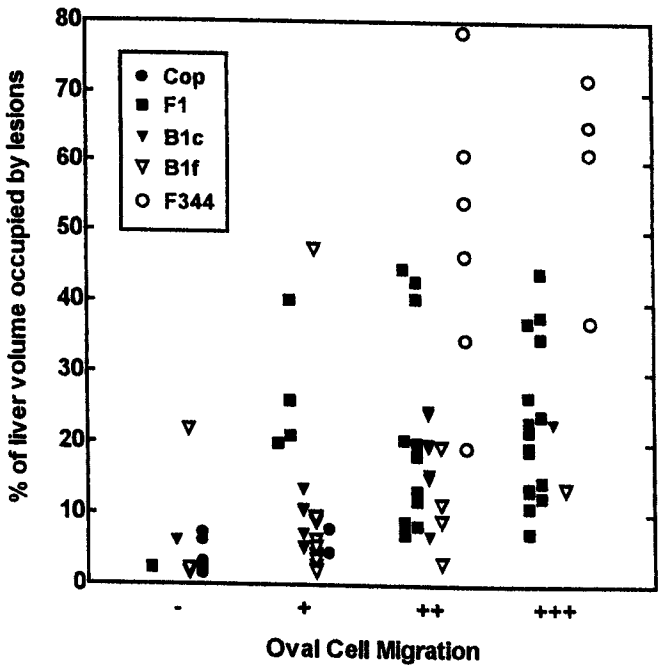

Figure 3. Extent of oval cell migration versus the percentage of liver volume occupied by GST 7-7-positive lesions for individual rats. Rats in the - category had the least oval cell migration from the portal areas and rats in the +++ category had the most migration.

were not further subdivided based on the strain of their granddams.

As shown in Figure 1, F1 rats are of intermediate resistance, having less liver volume occupied by lesions than F344 rats, but more than Cop rats. B1c rats have a susceptibility that is in between F1 and Cop rats, but not significantly different from either of these groups. Somewhat surprisingly, the susceptibility of B1f rats is not between F1 and F344 rats, and they are clearly more resistant than F1's.

Since the backcross generations are not genetically homogenous like their parents, we have plotted data from the individual rats to look for evidence of different phenotypes within the groups (Fig. 2). The Cop group has a narrower distribution than any of the other groups. The one F344 rat with ~20% of its liver volume occupied by lesions is surprisingly resistant, but is not a statistical outlier. Without very large numbers of individuals, it is difficult to separate a group into more than one phenotype. One individual rat in the B1f group, however, appears to have a phenotype that is clearly more susceptible than the rest of the group.

Oval Cell Migration. Previously, we found that the extent of oval cell migration is less in Cop than in F344 rats (6). Similarly, in the current experiment, rats with a small percentage of their liver volume occupied by lesions tended to have less extensive oval cell migration than rats with a large percentage volume occupied by lesions (Fig. 3). From this figure it is also apparent that there are some rats in which this relationship does not hold. Also, the F1 group and both backcross groups had individuals in each category of oval cell migration, whereas the Cop and F344 rats had low and high levels of oval cell migration respectively, as we have found previously (6).

Discussion

Initiation using DEN followed by promotion using the RH protocol is a well-established model of liver carcinogenesis in rats that has consistently been shown to produce many large GST 7-7-positive lesions in susceptible rats (15, 20). In the months following promotion, HCCs develop from a subset of these lesions (21). Previously, we have shown that Cop rats are resistant to the promotion of GST 7-7-positive liver lesions using the RH protocol, having ~10-fold less liver volume occupied by lesions than F344

rats 3 weeks following treatment (6). In the present study, resistant Cop rats and susceptible F344 rats were bred to produce F1 and backcross rats that were treated using the RH protocol in order to study the inheritance pattern of resistance and estimate the number of genetic loci involved.

Using the percentage liver volume occupied by lesions as a measure of susceptibility, our results show that F1 rats are more resistant than the F344 parental strain, but less resistant than Cop rats, indicating that resistance (or susceptibility) is incompletely dominant. Given the level of resistance of the F1 population, it might be expected that the B1c rats would have a mean resistance in between Cop and F1 rats and, indeed, this is what was observed (Fig. 1). By the same reasoning, the B1f population would be expected to be more susceptible than F1 rats, but more resistant than F344 rats, yet this is clearly not the case (Fig. 1). There is only one B1f rat that fits into this category and many of the other B1f rats are as resistant as Cop rats (Fig. 2). Although this one susceptible B1f rat could be an outlier, its presence, as well as the surprisingly high resistance of the some of the B1f rats, can be accounted for by a model of inheritance involving a minimum of four loci, with one locus showing recessive epistasis.

In a simple two-locus model with incomplete dominance, one-fourth of B1f rats would have a genotype identical to an F344 at these two loci, and would have the same level of susceptibility (Table I a). The rest of the B1f rats would be heterozygous at one or both of the loci and might be more similar to F1 rats. This two-locus model does not fit well with our observations since it predicts that five or six of the B1f rats would have an F344 phenotype. A four-locus model predicts that one-sixteenth of B1f rats would have an F344 phenotype, which is much closer to the 1/23 that we actually observed. Five- and six-locus models would predict that 1/25 and 1/36 B1f rats, respectively, would have an F344 phenotype. Although these models are consistent with our observation of the one susceptible B1f rat, we cannot test their goodness of fit using a chi-square or Fisher's exact test, since these tests require a minimum of five individuals in each category. In the groups of rats that have sufficient numbers, an analysis of expected versus observed outcomes would require each rat to be categorized phenotypically as susceptible, intermediate, or resistant. Since F1 rats are genetically homogenous, they must be placed into a single category of susceptibility, yet they are clearly phenotypically diverse (Fig. 2). Their range of susceptibility overlaps with all other groups and makes assignment of individuals from the backcross generations into categories impossible except for the one highly susceptible B1f rat discussed above.

In order to account for the many highly resistant B1f rats, a further refinement to the models must be made. Using the four-locus model as an example, of the 15/16 B1f rats that are not like an F344, 1/16 would have the same genotype as an F1. The remaining 14/16 would be homozygous for an F344 susceptibility allele at least at one locus that might be expected to make them at least as susceptible as an F1. However, this does not fit with the experimental data since some of these rats are highly resistant, while others are similar to F1 rats (Fig. 2). A genetic model that fits well with our observations includes recessive epistasis at one of the loci. In our four-locus model, one locus (for example 'D' in Table I b) confers some resistance when it is present in the heterozygous or homozygous dominant form, but is epistatic to the other loci in the homozygous recessive form (dd) and confers a high degree of resistance. One-half of the above mentioned 14/16 B1f rats would be heterozygous at this locus and would be like F1 rats, and the other one-half would be homozygous recessive and, due to epistasis, would be highly resistant. Although the one out of sixteen B1f rat that is like an F344 is homozygous recessive for this epistatic allele, this rat would still be susceptible since it has the same genotype as susceptible F344 rats (i.e., this allele would not be epistatic when the rat is homozygous recessive at all the other loci). Although this model predicts our experimental data well, the five- and six-locus models with recessive epistasis also fit well with our observations. Many more rats, however, would be required to test statistically which of these models best describes the inheritance of resistance in Cop rats.

In experiments similar to those described here, the re-

Table I. Contributing Gametes, Genotypes, and Predicted Phenotypes for a Two-Locus Model of Inheritance of Resistance in F1 × F344 Rats

F1	F344		Blf
	a	b	Phenotypes
A B	Aa	Bb	Intermediate
A b	Aa	bb	Intermediate
a B	aa	Bb	Intermediate
a b	aa	bb	Susceptible

F1	F344				Blf
	a	b	c	d	Phenotypes
A B C D	Aa	Bb	Cc	Dd	Intermediate
A B C d	Aa	Bb	Cc	dd	Resistant
A B c D	Aa	Bb	cc	Dd	Intermediate
A B c d	Aa	Bb	cc	dd	Resistant
A b C D	Aa	bb	Cc	Dd	Intermediate
A b C d	Aa	bb	Cc	dd	Resistant
A b c D	Aa	bb	cc	Dd	Intermediate
A b c d	Aa	bb	cc	dd	Resistant
a B C D	aa	Bb	Cc	Dd	Intermediate
a B C d	aa	Bb	Cc	dd	Resistant
a B c D	aa	Bb	cc	Dd	Intermediate
a B c d	aa	Bb	cc	dd	Resistant
a b C D	aa	bb	Cc	Dd	Intermediate
a b C d	aa	bb	Cc	dd	Resistant
a b c D	aa	bb	cc	Dd	Intermediate
a b c d	aa	bb	cc	dd	Susceptible

Note. In this model, F344 and Cop parental strains are homozygous susceptible (lower case) and homozygous resistant (upper case), respectively, at all loci. (b) is similar to (a), but with a four-locus model and recessive epistasis of the d allele.

sistant BN rat strain has been crossed to yield F1's and backcrosses to F344s (13). In these experiments, the percentage of liver volume occupied by lesions in the backcrosses varied widely. Similar to our results, some of the backcrosses were as resistant as the BN rats, although a much higher proportion of the backcross rats were as susceptible as the F344 rats. Five loci potentially involved in BN resistance were identified (13).

Treatment of rats with the RH protocol is known to stimulate proliferation and migration of oval cells that originate in portal areas (7). Previously, we have shown that Cop rats have less oval cell migration compared with F344 rats (6). In the current study we found a similar trend such that rats with smaller percentage of liver volumes occupied by lesions had less oval cell migration, regardless from which group they came (Fig. 3). This relationship, however, did not hold for all rats, since some had extensive oval cell migration and a small percentage of volume occupied by lesions or visa versa. This would suggest that the migration of oval cells is neither necessary nor sufficient for the development of large liver lesions, but may be associated with lesion growth.

In summary, we have found that resistance to the promotion of preneoplastic lesions in Cop rats is a complex, polygenic trait that is incompletely dominant. We can best account for the observed phenotypes in the F1 and backcross generations by a minimum of four loci, one of which shows recessive epistasis.

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