Incorporating Mouse Genome
Mammalian Genome
Genes and Phenotypes

Further studies on using multiple-cross mapping (MCM) to map quantitative trait loci

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Abstract

We have completed whole-genome scans for quantitative trait loci (QTLs) associated with acute ethanol-induced activation in the six F_2 intercrosses that can be formed from the C57BL/6J (B6), DBA/2J (D2) , BALB/cJ (C), and LP/J (LP) inbred strains. The goal was to test the hypothesis that given the relatively simple structure of the laboratory mouse genome, the same QTLs will be detected in multiple crosses which in turn will provide support for the strategy of multiple-cross mapping (MCM). QTLs with LOD scores greater than 4 were detected on Chrs 1, 2, 3, 8, 9, 13, 14, and 16. Only for the QTL on distal Chr 1 was there convincing evidence that the same or at least a very similar QTL was detected in multiple crosses. We also mapped the Chr 2 QTL directly in heterogeneous stock (HS) animals derived from the four inbred strains. At G_{19} the QTL was mapped to an approximately 3-Mbp interval and this interval was associated with a haplotype block with a largely biallelic structure: B6-L:C-D2. We conclude that mapping in HS animals not only provides significantly greater QTL resolution, at least in some cases it provides significantly more information about the QTL haplotype structure.

Introduction

Our use of multiple cross mapping (MCM) has undergone a marked evolution. Initially, completely unrelated F_2 intercrosses were used to detect multiple quantitative trait loci (QTLs) that we predicted would be related by influencing common genetic networks (Patel and Hitzemann 1999; Rasmussen et al. 1999). The next stage integrated the QTL information from these unrelated intercrosses with the haplotype structure found in genetic maps (Hitzemann et al. 2000, 2002, 2003). This phase began with the recognition that the uneven distribution of polymorphic microsatellite markers across the genome was not random and rather reflected the distribution of domains that were conserved and not conserved among the standard laboratory mouse strains (Hitzemann et al. 2000). This mosaic structure of the genetic map was not unexpected because the common laboratory mouse strains are actually derived from a limited number of founders (Bonhomme 1986; Silver 1995; Beck et al. 2000). Subsequently, Wade et al. (2002) were able to show that the common laboratory strains were actually largely derived from two original subspecies (*Mus mus domesticus* and *M. m. musculus*) with a limited contribution from *M. m. castaneus*. We proposed that the multiple-cross QTL information could be used to develop an empirical algorithm for sorting the microsatellite data to detect regions with the highest probability of containing a QTL (Hitzemann et al. 2000). The underlying principle of this analysis was that the structure of the genetic map provided information and, thus, statistical power which would enhance QTL analysis and potentially significantly reduce the QTL interval.

Although our earliest application of the multiple-cross approach was viewed as moderately successful (Hitzemann et al. 2000), we recognized that the approach was flawed in that important ''cross'' data were missing. For example, consider the following situation: A Chr 1 QTL for ethanol-induced locomotor activation is found in a C57BL/6J (B6) × DBA/2J (D2) intercross (Demarest et al. 1999,

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2001) but not in a BALB/cJ (C) × LP/J (LP) intercross (Hitzemann et al. 2000). The simplest interpretation of these data is that the B6 and D2 strains are polymorphic at the QTL, whereas the C and LP strains are not. However, we have no idea if the QTL would be generated by crossing the B6 or D2 strains with the C and LP strains. This additional information, when entered into the sorting algorithm, should have the effect of markedly improving QTL resolution. To test this hypothesis, we constructed a balanced panel of six F_2 intercrosses derived from the B6, D2, C, and LP progenitor strains and used these crosses to map QTLs for the acute ethanol response (Hitzemann et al. 2002). The multiple-cross QTL data were used to narrow a Chr1 QTL to a 3-cM interval that was confirmed by mapping in heterogeneous stock (HS) animals (Talbot et al. 1999).

Our next step was to use MCM as a strategy to integrate QTL, gene expression, and sequence analyses (Hitzemann et al. 2003). A similar approach was developed independently by Park et al. (2003). The earliest proof of principle (in rodent models) for the integration of QTL and gene expression data were two studies that identified genes involved in insulin resistance (Aitman et al. 2000; Collison et al. 2000) and airway hyperresponsiveness (Karp et al. 2000). The first application of the approach to neural phenotypes is found in Sandberg et al. (2000) who reported marked differences in brain gene expression between two inbred mouse strains, B6 and 129S1/SxImJ, in whole-brain and discrete brain regions (cortex, midbrain, hippocampus, and cerebellum). These data led the authors to the salient observation that some of these differences appeared to coincide with the known location of ''behavioral'' QTLs; a particular note was made of the fact that *Kcnj9* (which encodes GIRK3, a G-protein-coupled inwardly rectifying potassium channel) had a markedly lower expression in the B6 strain and was located in a QTL-rich region on Chr 1 (see, e.g., Flint 2003). Subsequent publications (Carter et al. 2001; Lockhardt and Barlow 2001, but also see Gerschwind 2000; Belknap et al. 2001; Flint and Mott 2001; Mackay 2001; Wayne and McIntyre 2002; Hitzemann et al. 2003, 2004; Cervino et al. 2005; Chesler et al. 2005) have continued the argument and provided additional arguments for combining analyses of transcript levels (using expression arrays) with information from QTL mapping to nominate candidate genes.

The current study continues our investigation of MCM. We have completed the genome-wide scan for ethanol response in all six intercrosses and have identified all the major QTLs (here defined as those QTLs associated with approximately 5% or more of the phenotypic variance). A key goal of these experiments was to determine the proportion of QTLs that had multiple signatures across the six intercrosses. If a significant number of the QTLs were detected in multiple crosses, this would provide strong support not only for the MCM strategy but also for the related strategy we have termed multiple-strain mapping (MSM) (see, e.g., Grupe et al. 2000; Wade et al. 2002). We also have considered that a more efficient variant of MCM would be to map the QTLs directly in a four-way cross formed from the B6, D2, C, and LP strains, the same progenitors used to derive the MCM crosses. The key issue of concern was whether we could extract the same QTL signature from the four-way cross as was obtained from the six F_2 intercrosses. The four way cross, hereafter termed heterogeneous stock 4 (HS4), was interrogated at G_5 and G_{19}.

Methods

Animals. Male and female B6, D2, C, and LP animals were obtained from The Jackson Laboratory (Bar Harbor, ME). Reciprocal F_1 hybrids were formed, which in turn were bred to form the F_2 intercross animals. Approximately equal numbers of male and female animals for each cross were phenotyped; *N* ranged from 550 to 600 per cross, which is sufficient to detect a QTL accounting for 4%–5% of the trait variance with greater than 80% power (Lander and Kruglyak 1995). The formation of the HS4 colony preceded the development of the intercrosses such that the G_5 animals were tested under conditions identical to the intercrosses. To develop the HS4, the 12 different reciprocal F_1 hybrids were mated to produce 24 unique crosses. The number of G_2 families was doubled and the resulting 48 families were maintained in a circle breeding design. The HS4 animals were tested for ethanol-induced activity at G_5 and G_{19}. All animal care, breeding, and testing procedures were approved by the Laboratory Animal Users Committees at the Veterans Affairs Medical Center, Portland, OR, and the Oregon Health & Science University, Portland, OR.

Measurement of ethanol response. Mice were removed from the home cage, injected with saline (10 ml/kg), and placed individually in the testing arena; the arena floor was covered with standard laboratory bedding. Activity was monitored for 20 min under standard laboratory lighting conditions. The following day animals were administered 1.5 g/kg ethanol and the activity measurements repeated. The ethanol response is the difference in activity between days 2 and 1 (reported as distance

[cm] traveled). One week later the test was repeated and the two ethanol responses were averaged (Markel et al. 1995). Locomotor activity was assessed in a San Diego Instruments Flex Field locomotor system. The apparatus comprised a 4 × 8 array of photocells mounted in a 25 × 47-cm metal frame, situated 1 cm off the floor, and surrounding a 22 × 42 × 20-cm high plastic arena. Activity was recorded over eight 2.5-min blocks.

DNA isolation. High-molecular-weight genomic DNA was isolated from liver samples as follows: 250–500 mg liver tissue was minced with a sterile razor blade, transferred to a 15-ml polypropylene Falcon tube with 5 ml lysis buffer [100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 100 μg/ml proteinase K, 200 mM NaCl], and incubated with rocking at 55°C overnight. After incubation, 20 μl/ml of 5M NaCl was added with gentle inversion. The tissue digest was extracted twice with equilibrated phenol, once with equal volumes of phenol and chloroform:isoamyl alcohol (chisam) (24:1) and once with chisam alone. DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of ice cold ethanol. Dried DNA pellets were resuspended in double-distilled water (ddH_2O). Purity and concentration of the final samples were evaluated by ultraviolet (UV) spectroscopy and only samples with a 260/280 ratio > 1.4 were used for genotyping.

Genotyping the microsatellite polymorphisms. All of the microsatellite genotyping involved the -(CA)–repeating microsatellites (Dietrich et al. 1992). The PCR primer sets were obtained from Invitrogen. Genomic DNA (1–5 ng) was amplified with 18 pmol of each primer, 0.5 U of Taq polymerase (AmpliTaq, PerkinElmer Cetus, or Taq DNA polymerase, Boehringer Mannheim Biochemica), and 100 nM dNTPs in a 20-μl reaction under the standard conditions recommended by the manufacturer. All reactions were amplified in a PerkinElmer Thermal Cycler. Products were visualized by electrophoresis in 1 × TBE buffer on a 3% agarose gel (3:1 Metaphor agarose, FMC Bioproducts). Bands were visualized by ethidium bromide staining.

Genotyping single nucleotide polymorphisms (SNPs). A database of SNPs polymorphic among the four strains was obtained from R. Mott (Oxford) with the permission of T. Wiltshire, Genetics Institute of the Novartis Research Foundation (GNF). These SNPs were abstracted from a larger database described in Wiltshire et al. (2003) and had been found to work well in the Illumina SNP platform. DNA samples from the HS4 animals at G_{19} only and meeting the Illumina Q/C standard were sent to the company for analysis. Two sets of controls for the ten possible genotypes were imbedded in the sample set; all were correctly identified.

Results

Characteristics of the ethanol response. Details of the ethanol response in the four progenitor strains have been described elsewhere (Demarest et al. 2001) and will not be described here except to note that the D2 and C strains show a net locomotor excitatory response to the 1.5 g/kg ethanol challenge, while the B6 and LP strains show a mild-to-moderate net inhibition of activity. Some details of the ethanol response among the six F_2 populations are found in Hitzemann et al. (2002). Additional details on the F_2 populations and data on the HS4 cross are found in Fig. 1. To simplify the data analyses and given the circle breeding design used to maintain heterozygosity (minimize inbreeding), the HS4 at G_5 and the HS4 at G_{19} were treated as independent samples. The ethanol response data have been collapsed into two time intervals, 0–5 and 5–20 min because this convention effectively demonstrates the main differences among the crosses over time. The average saline, ethanol, and difference score data for the 0–5-min interval are illustrated in Fig. 1. The analysis of variance (ANOVA) for the 0–5-min data revealed a significant treatment (saline vs. ethanol challenge) × cross interaction ($F_{7,\ 9499} = 98$, $p < < 10^{-20}$). The *post hoc* analysis (Tukey's HSD) indicated that the saline versus ethanol challenge data were significantly different ($p < 0.001$ or better) for all crosses except the B6 × C ($p > 0.45$) and LP × B6 ($p > 0.99$). The ANOVA for the 0–5-min difference score was significant for cross ($F_{7,\ 4750} = 187$, $p < <10^{-20}$).

The ANOVA for the 5–20-min data also revealed a significant treatment × cross interaction ($F_{7,\ 9499} = 41$, $p <<10^{-20}$) (Fig. 1). The *post hoc* analysis indicated that the saline versus ethanol challenge data were significantly different ($p < 0.001$ or better) for all crosses except the B6 × C ($p > 0.12$) and D2 × C ($p > 0.80$). The ANOVA for the 5–20-min difference score was significant for cross ($F_{7,\ 4750} = 62$, $p < <10^{-20}$). The *post hoc* analysis indicated that among the three crosses that showed an ethanol-induced decrease in activity, there were no significant differences. Furthermore, there was no significant difference in the HS4 G_5 and HS4 G_{19} data ($p > 0.99$).

The variances for the 0–5- and 5–20-min data are also illustrated in Fig. 1. The number of animals in each of the crosses varied from 550 to 610. A

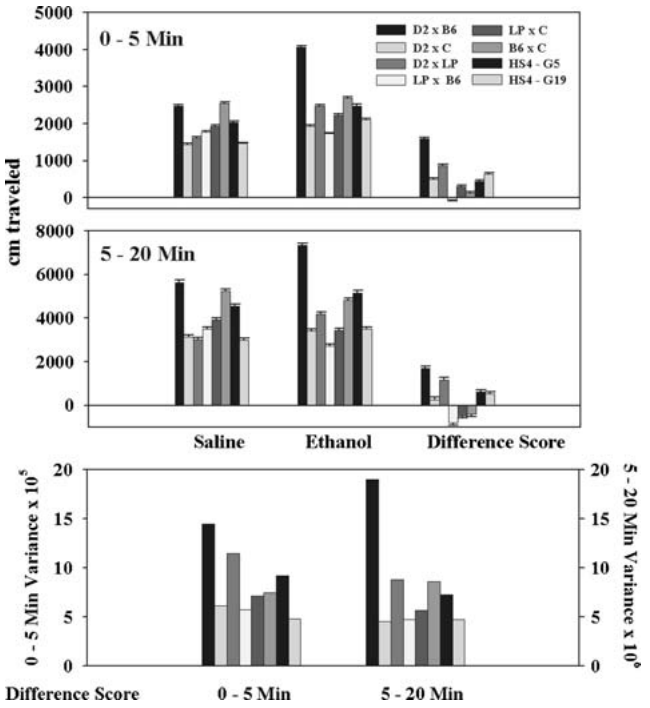

Fig. 1. Characteristics of the ethanol response in the six F_2 intercrosses and the HS4 animals. The ethanol response phenotype has been described in detail elsewhere (Demarest et al. 1999, 2001). Briefly, animals are administered saline (10 ml/kg) on day 1 and activity is immediately monitored for the next 20 min. On day 2, the procedures are the same except that the animals are administered 1.5 g/kg ethanol. The ethanol response is the difference score (day 2-day 1). The entire procedure is repeated one week later; the results from the two trials are averaged (Markel et al. 1995). Data are presented for the average saline, ethanol, and difference scores; data were collapsed into two intervals: 0–5 and 5–20 min. Data are presented as the mean cm traveled ± SE. The variances for the two time intervals are shown in the bottom graph. n = 550–610.

difference in variance of more than 1.25-fold is significant at $p < 0.005$. The variance was greatest in the D2 × B6 cross and lowest in the crosses between the phenotypically similar inbred strains (D2 × C and LP × B6). A significant decrease in variance was noted in the HS4 G_{19} compared with the HS4 G_5 animals; the decrease for the 0–5- and 5–20-min intervals was 48% and 35%, respectively.

QTL Analysis in the F_2 intercrosses. The computer program R/qtl (Broman et al. 2003) was used within the R statistical programming environment (http://www.r-project.org). The EM (expectation maximization) option was used to attain 5-cM interval mapping. For all of the F_2 crosses, the genome-wide scan began with genotyping the phenotypic extremes (top and bottom 16%). The ethanol response phenotype was parsed into 2.5-min intervals and the extreme individuals for each interval were genotyped. As noted below, this parsing of the data provided an important temporal QTL signature. Although each of the intervals was significantly correlated, the total number of animals genotyped during the discovery phase routinely was in the range of the extreme 20%–25% to include the extreme 16% for each interval. From R/qtl, the genome-wide single-trait LOD threshold for a suggestive and significant QTL was 2.3 and 3.7, respectively. We recognized that given the multiple (albeit non-independent) tests being performed for each cross and given that six crosses and the HS4 animals were analyzed , a multiple-test adjustment would normally be required for the detection of a single QTL. However, given that our goal was to determine the likelihood of a QTL being present in multiple crosses, we took the LOD threshold of 3.7 as being sufficient to warrant a more detailed investigation. For QTLs meeting this threshold, all animals were genotyped within the relevant cross and additional markers were added in the region of interest to confirm the presence of the QTL. If an apparently similar QTL was detected in another cross at the suggestive threshold, the same followup procedures were applied.

Figures 2 and 3 illustrate the peak LOD scores obtained in the six F_2 intercrosses at each locus, independent of time interval. In addition to the QTLs previously reported on Chrs 1 and 2 (Demarest et al. 1999, 2001; Hitzemann et al. 2002), two QTLs with LOD scores of greater than 6 were detected on Chr 13 (LP × B6) and Chr 15 (B6 × C). QTLs with LOD scores ranging from 4 to 6 were detected on Chr 3 (D2 × C), Chr 8 and 9 (LP × B6) and Chr 14 and 16 (LP × C). On Chr 14, the data point to the presence of two QTLs. Only the QTL on Chr 1 was confirmed in a related cross with a LOD score exceeding the nominal threshold of 3.7. Reducing the LOD threshold for confirmation to greater than 2.3 but less than 3.7, potential confirmation was found for the QTLs on Chrs 1, 3, 8, 9, 13, and 16.

Three criteria in decreasing order of importance were adopted for determining QTL similarity: (1) a parallel genotype × phenotype relationship associated with the peak LOD , i.e., an allele-specific effect; (2) a parallel temporal peak LOD pattern; and (3) a parallel peak LOD location. QTL similarities on Chrs 8, 13, and 16 were immediately rejected on the basis of the genotype × phenotype relationship, e.g., on Chr 8, and for the LP × B6 intercross, increased activity was associated with the LP strain, while for the LP × D2 strain, increased activity was associated with the D2 strain. Details of the tests for QTL similarity on Chrs 1, 3, and 9 are found in Figs. 4–6.

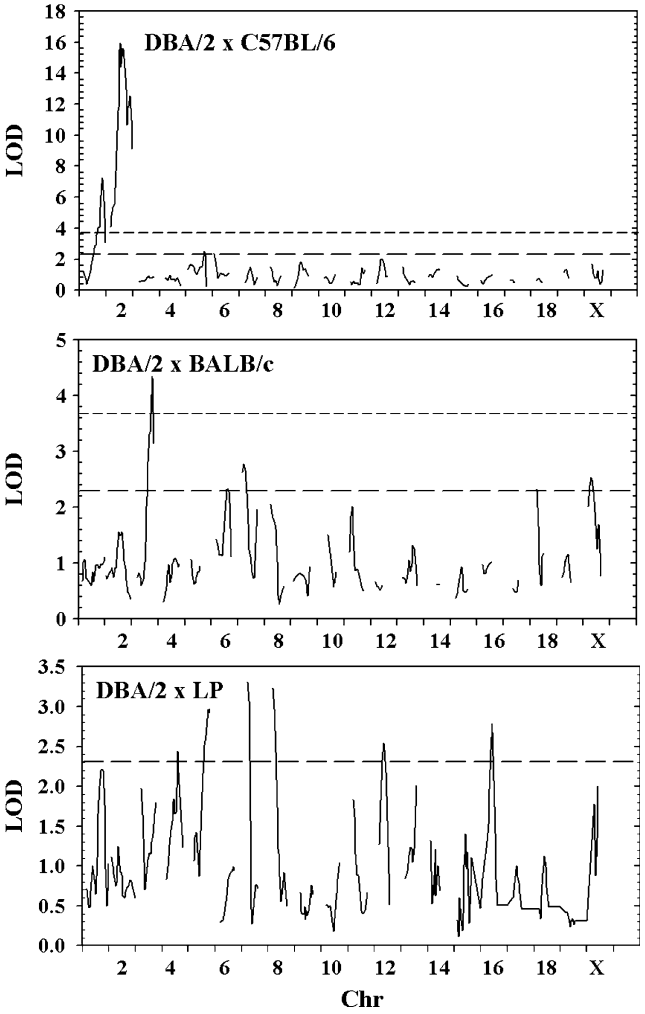

Fig. 2. Genome-wide distribution of peak LOD scores in the DBA/2J × C57BL/6J, × BALB/cJ, and × LP/J intercrosses. QTL analysis was performed for each 2.5-min interval as described in the Results section. The graphs plot the peak LOD score obtained, regardless of time. From R/qtl (Broman et al. 2003), the genome-wide single-trait LOD threshold for a suggestive and significant QTL is 2.3 and 3.7, respectively; these thresholds are indicated in the graphs.

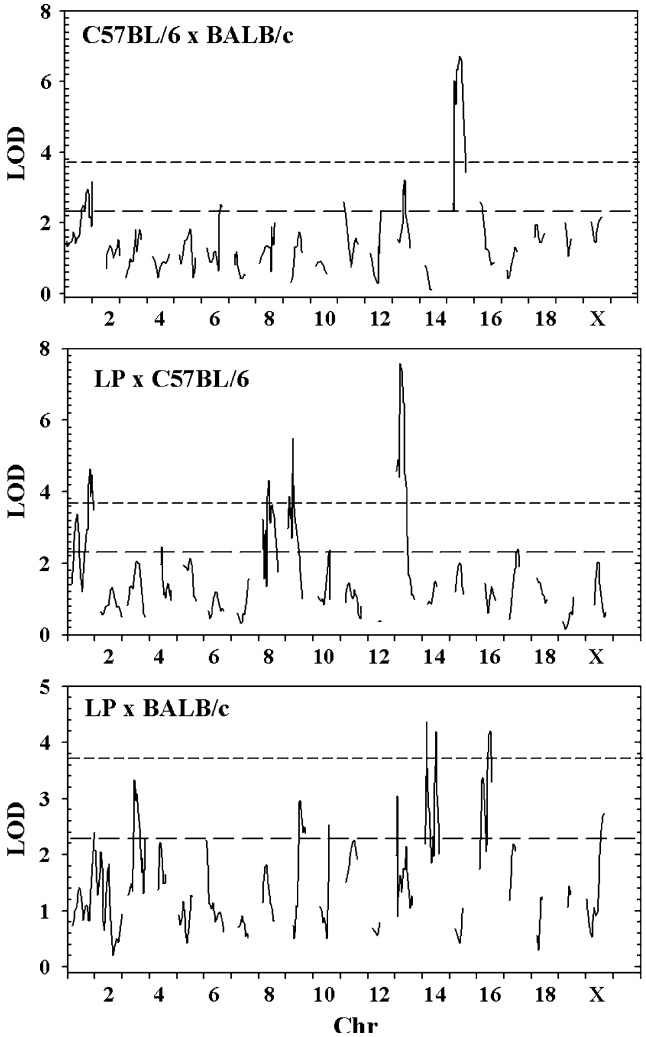

Fig. 3. Genome-wide distribution of peak LOD scores in the C57BL/6J × BALB/cJ and LP/J × C57BL/6J and × BALB/cJ intercrosses. Details are the same as in the legend to Fig. 2.

On Chr 1 the peak LOD value (7.3) in the primary intercross (D2 × B6) (a) was found on distal Chr 1and (b) was associated with the 2.5–5-min interval (Fig. 4). A similar QTL was found in the LP × B6 intercross (peak LOD = 4.6) with essentially identical characteristics; there was also suggestive evidence in this cross for a more proximal QTL (peak LOD = 3.4). For the C × B6 intercross, the peak LOD score in the region of interest (peak LOD = 3.1) was only suggestive and was not significantly different from the LOD values obtained across the entire chromosome. The genotype × phenotype relationship for the three intercrosses was assessed at *D1Mit150*, which has an estimated position of 100 cM. ANOVA revealed that all three crosses showed a significant genotype × time interaction ($p < 10^{-4}$ or better); the *post hoc* analysis indicated that the most significant ($p < 5 \times 10^{-3}$ or better) genotype effect for all three intercrosses was found for the 2.5–5-min interval. This interval is associated with the "explosive" activation response to ethanol and is known not to be associated with differences in ethanol metabolism (Phillips et al. 1995). The three intercrosses showed a similar pattern in that the ethanol response was highest for the B6:B6 genotype.

Figure 5 illustrates that on Chr 3 the peak LOD score (4.3) in the primary intercross (D2 × C) (a) was found on distal Chr 3 and (b) was associated with the 2.5–5-min interval; the genotype × phenotype interaction illustrated that the C strain allele was associated with increased activity. A somewhat similar but only suggestive QTL (peak LOD = 3.3)

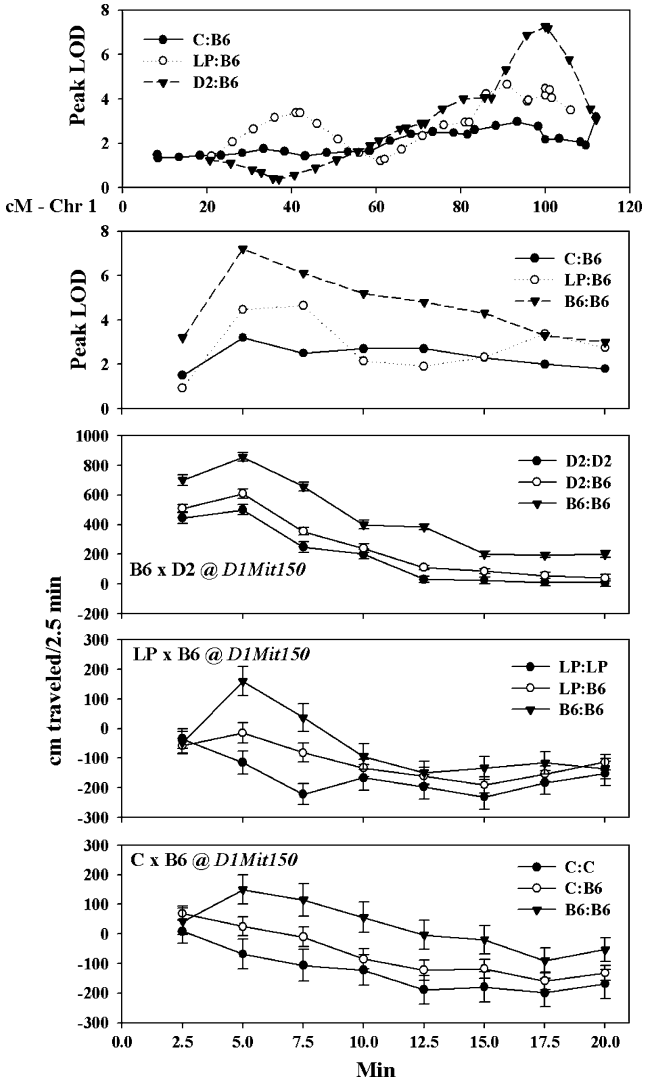

Fig. 4. Similarity of the QTL for ethanol response on Chr 1 in three different F_2 intercrosses: DBA/2J × C57BL/6J (D2 × B6), C57BL/6J × BALB/cJ (B6 × C), and LP/J × C57BL/6J (LP × B6). The bottom three graphs plot the genotype × phenotype interaction for the three intercrosses; the genotypic data for each intercross were obtained using the same marker, *D1Mit150*. The marker is found at 174.5 Mbp and is located in an intronic region of formin 2 (*Fmn2*). ANOVA revealed that all three crosses showed a significant genotype × time interaction ($p < 10^{-4}$ or better); the *post hoc* analysis indicated that the most significant ($p < 5 \times 10^{-3}$ or better) genotype effect for all three intercrosses was found for the 2.5–5-min interval. The peak LOD score for each time interval in each of the intercrosses is also plotted. The top graph shows the distribution of LOD scores on Chr 1 in the three intercrosses; data are for the 2.5–5-min interval.

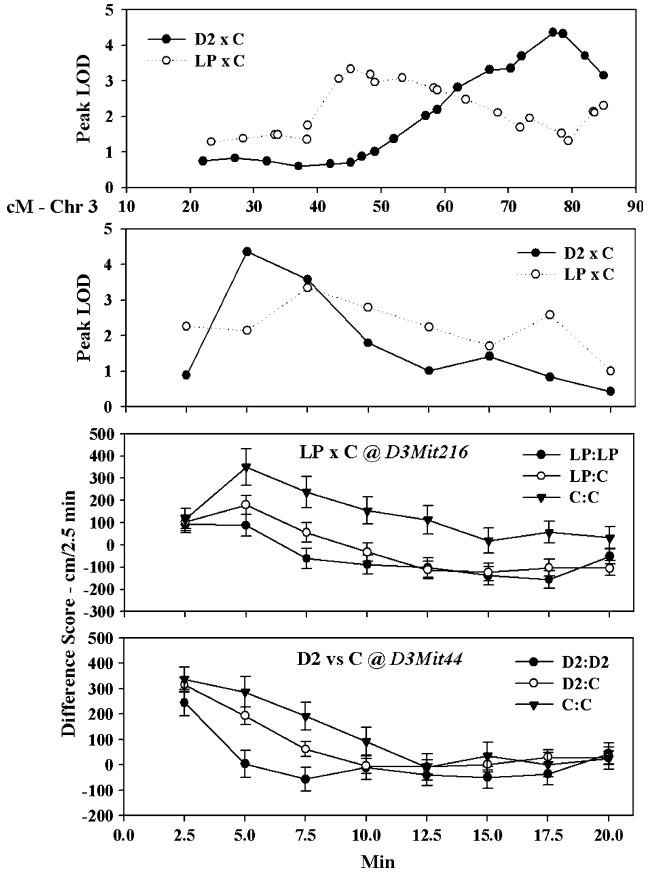

Fig. 5. Similarity of the QTL for ethanol response on Chr 3 in two different F_2 intercrosses: DBA/2J × BALB/cJ (D2 × C) and LP/J × BALB/cJ (LP × C). The details are similar to those presented in the legend to Fig. 4. *D3Mit44* and *D3Mit216* are found on Chr 3 at 147.1 and 122.2 Mbp, respectively; these are the markers most closely associated with the peak LOD scores in the two intercrosses. At *D3Mit17* (142.6 Mbp), the associated LOD score in the LP × C intercross was 1.3.

was detected in the LP × C intercross. Both the genotype × phenotype relationship and the temporal position of the peak LOD score was generally consistent with the data from the D2 × C intercross. The QTLs appeared to be different in that the position of the peak LOD in the LP × C intercross appeared to be more distal; however, the 95% confidence intervals (CI) for the peak LOD values were quite broad and overlapped (data not shown).

The Chr 9 QTL in the LP × B6 intercross (Fig. 6) differed from those on Chrs 1 and 3 in that the peak LOD scores were associated with a broader time interval (5–20 min). Using the 5–20-min data as a combined phenotype resulted in a somewhat improved peak LOD score (5.4 vs. 4.5) (data not shown). The QTL was broadly distributed across proximal Chr 9 (data illustrated for the 5–7.5-min interval) and an ethanol-induced inhibition of activity was associated with the LP:LP genotype. The suggestive QTL in the LP × C intercross was similar to the LP × B6 QTL in that the ethanol response was lowest in LP:LP genotype. However, it differed in that the threshold for a suggestive QTL was met for only a single time point (2.5–5 min) and the position of

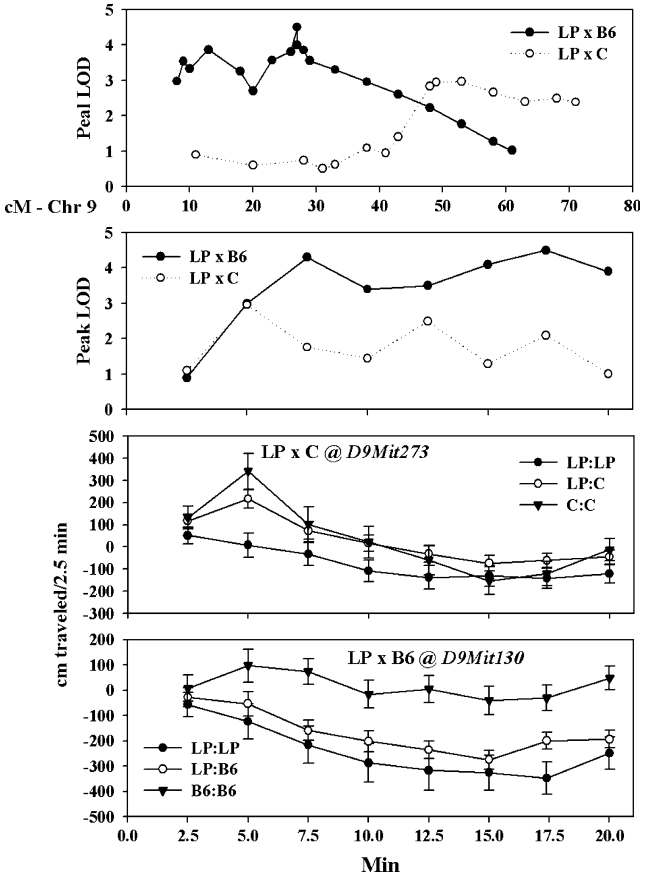

Fig. 6. Similarity of the QTL for ethanol response on Chr 3 in two different F_2 intercrosses: LP/J × C57BL/6J (LP × B6) and × BALB/cJ (LP × C). The details are similar to those presented in the legend to Fig. 4. *D9Mit330* and *D9Mit273* are found on Chr 9 at 47.1 and 92.4 Mbp, respectively; these are the markers most closely associated with the peak LOD scores in the two intercrosses.

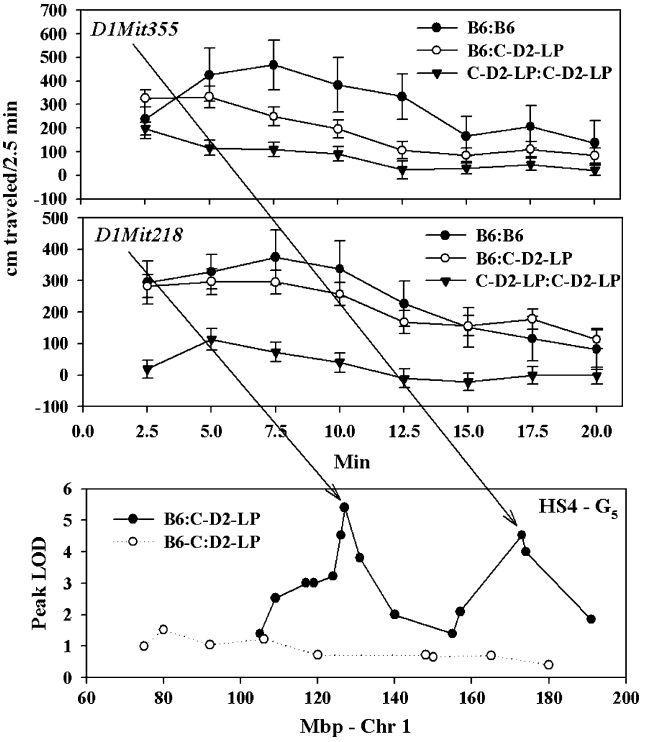

Fig. 7. Mapping QTLs for ethanol response on distal Chr 1 in heterogeneous stock 4 (HS4) animals at generation 5 (G_5). Peak LOD scores are presented in the lower graph for two contrasts: B6 × (D2-C-LP) and (B6-C) × (D2-LP); the analysis was reduced to a biallelic contrast. The data suggest that there may be two QTLs present. The genotype × phenotype interaction is presented for the two markers associated with the peak LOD scores. *D1Mit128* and *D1Mit355* are found at 127.3 and 173.3 Mbp, respectively.

the QTL appeared to be more distal on Chr 9, although again it needs to be noted that the 95% CI for these QTLs were quite broad.

QTL analysis in HS4 animals. At G_5 or three generations after forming the four-way cross, the HS4 animals were phenotyped for ethanol response and genotyped in the regions of the known QTLs on Chrs 1 and 2. The genotyping strategy involved reducing the microsatellite-based analysis to diallele contrasts, e.g., B6 vs. (C-D2-LP) [B vs. CDL]. For Chr 1, the following contrasts were examined in some detail: B6 × C-D2-LP, B6-LP × C-D2, B6-C × D2-PL, B6-C-LP × D2, and B6-D2 × C-Lp. Significant LOD scores were obtained for only the B6 × C-D2-LP contrast (Fig. 7); for comparison, the B6-C × D2-LP contrast is also illustrated. The data in Fig. 7 suggest that two QTLs may be present on distal Chr 1; for both of the apparent QTLs, the B6 allele is associated with increased activation. On Chr 2, we observed that two contrasts (B6 vs. C-D2-LP and B6-LP vs. C-D2) produced significant LOD scores (Fig. 8), the LOD time course was similar, and for both contrasts the B6 allele was associated with a decreased response.

The Chr 2 QTL was further analyzed using a SNP-based analysis in HS4 animals at G_{19}; on average, the inter-SNP interval was 1 Mbp. The results obtained are illustrated in Fig. 9. The interval of interest (~103–130 Mbp) is characterized largely by two different biallelic SNP blocks: B6:C-D2-LP and B6-LP:C-D2. The QTL was clearly associated with the B6-LP:C-D2 block located between 112.5 and 117.5 Mbp.

Discussion

Combining data from multiple crosses to improve QTL characterization has been widely used in agricultural research. Elaborate algorithms have been developed to integrate the results from, in some cases, hundreds of crosses (Yi and Xu 2002; Khatkar

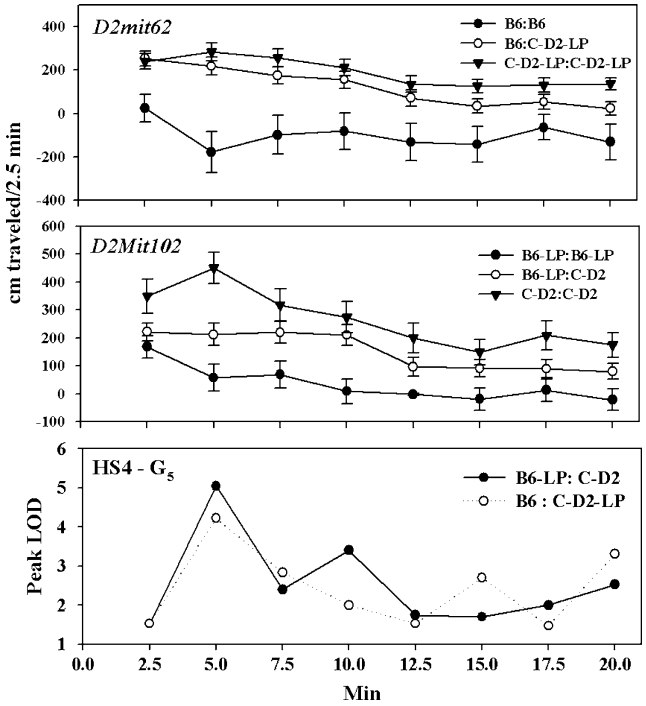

Fig. 8. Mapping QTLs for ethanol response on Chr 2 in heterogeneous stock 4 (HS4) animals at generation 5 (G_5). Peak LOD scores are presented in the lower graph for two contrasts: B6 × (D2-C-LP) and (B6-LP) × (D2-C). As in Fig. 7, the analysis was reduced to a biallelic contrast. Over the region of interest, both contrasts produced essentially equivalent results. *D2Mit62* and *D2Mit102* are found at 117.6 and 113.8 Mbp, respectively, and are the markers associated with the peak LOD scores for the two contrasts.

et al. 2004). The application of MCM to traits of physiologic relevance is relatively recent (Hitzemann et al. 2002; Park et al. 2003; Li et al. 2005); the

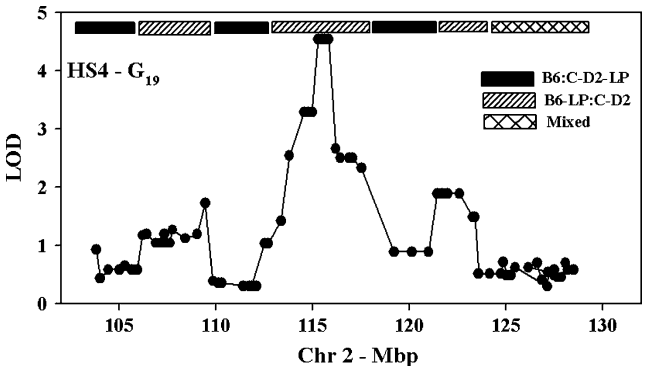

Fig. 9. Mapping the QTL for ethanol response on Chr 2 in heterogeneous stock 4 (HS4) animals at generation 19 (G_{19}). G_{19} animals (n = 600) were genotyped using a SNP-based analysis across the region of interest (~ 103–128 Mbp). The region is characterized largely by two biallelic blocks: B6:C-D2-LP and B6-LP:C-D2. The QTL peak is associated with B6-LP:C-D2 block that extends from 112.5 to 117.5 Mbp. LOD scores were calculated using a marker-by-marker based analysis.

increasing use of this approach parallels the development of detailed genetic maps, particularly microsatellite maps for laboratory animals (e.g., Dietrich et al. 1992, 1994, 1996). Flint et al. (1995) were the first to describe for a large F_2 intercross the QTL analysis of a behavioral trait (open-field activity). Subsequently, hundreds of behavioral QTLs have been detected in backcrosses and F_2 intercrosses, many with highly significant LOD scores (see, e.g., Flint et al. 2003). It was of interest to our laboratory to note the overlap of QTLs for behaviorally similar traits that were detected in genetically different mapping populations (Hitzemann et al. 2000). For example, an apparently similar QTL for open-field response was detected on distal Chr 1 in B6 vs.A, B6 vs. C, B6 vs. D2 and B6 × LP F_2 intercrosses (Gershenfeld et al. 1997; Flint et al. 1995; Koyner et al. 2000; Hitzemann et al. 2003). In particular, these data led us to conclude that by combining data from multiple crosses one could both improve QTL localization while at the same time determine the QTL signature (which approximates the haplotype structure of the QTL) (Hitzemann et al. 2000).

The primary question addressed in this report was the following: Using a completely balanced panel of F_2 intercrosses derived from four inbred laboratory mouse strains, how frequently would we detect the same QTL? Assuming (see Introduction) that the structure of the laboratory mouse genome is largely biallelic, there was a strong expectation that the same QTL would be detected in multiple crosses. The sample sizes used in the current study were sufficient to detect a QTL associated with 4%–5% of the phenotypic variance at a LOD of 3.7 or better and 80% power. The data in Figs. 2 and 3 illustrate that a total of ten QTLs exceeding the LOD threshold of 3.7 were detected in the six intercrosses; four of the QTLs (Chrs 1, 2, 13, and 16) had LOD scores of 6 or better. Three criteria in decreasing order of importance were used to estimate if similar QTLs were detected in multiple crosses: (1) the allele effect was identical, (2) the peak LOD showed a similar temporal pattern, (3) the peak LOD showed a similar location. We recognized at the outset that these criteria were not sufficient to conclude QTL identity; QTL identity would require demonstrating that the same polymorphism or polymorphisms are responsible for affecting the trait of interest. The criteria used were ones that we considered necessary to conclude that a *similar* QTL was found in multiple crosses. Of these criteria, the last one— similar QTL location, was the least accurate given the broad 95% CI associated with mapping in F_2 intercrosses. Of the ten QTLs, only the QTL on distal Chr 1 met

all three criteria and was detected in two different intercrosses (B6 × D2 and B6 × LP) with LOD scores of greater than 4. A similar QTL was also likely present in the B6 × C intercross, although the strength of the association was only suggestive and the peak LOD was quite broad. Dropping the LOD threshold to greater than 2.3, QTLs were detected in multiple crosses on Chrs 3, 8, 9, 13, and 16. Of these, the QTLs on Chrs 8, 13, and 16 were immediately rejected as not being similar given the marked differences in the allele effects. The match on Chr 3 met the criteria for the allele effect and the temporal pattern; the position of the peak LOD appeared to be different but, as noted above, this criterion is the least reliable. The QTLs on Chr 9 matched only for allele effect.

In retrospect, we now recognize that the third criterion (similar QTL location) probably should have been dropped and replaced by directly testing for a cross x genotype interaction while combining the results of various crosses. Elements of this approach are found in Li et al. (2005). However, to make this statistical approach work most efficiently would require a set of microsatellite markers that discriminate among all four strains. Among the *Mit* series of markers there are less than 300 that meet this criterion and these are not evenly distributed across the genome (unpublished observation). A workable alternative would be to perform a dense SNP genotyping of the animals, sufficient to infer the haplotypes associated with the four strains in each QTL region. Evidence supporting this approach is found in the data obtained with the HS4 animals (see below).

Even given the caveats noted above, we still conclude that detecting the same QTL in multiple crosses is not common and may in fact be relatively rare. However, the possibility cannot be excluded that a QTL is actually present in multiple crosses but was simply not detected. There are several potential reasons why this may have occurred. One, given the sizes of the mapping populations, relatively small-effect QTLs would not be detected. Two, a QTL may have been present in multiple crosses but the phenotypic effects were silenced in one or more crosses by the effects of modifier loci. The effects of genetic background on the expression of behavioral QTLs has long been recognized (e.g., Frankel et al. 1995). Gill et al. (2000) surveyed the ethanol activation QTLs detected in three different populations of recombinant inbred lines (BXD, AXB-BXA, and LSXSS). Of the 12 QTLs detected, overlap was found for only four. Similarly, while there is some overlap, there are also marked differences in ethanol preference QTLs detected in B6 × D2 and B6 × 129P3/J F_2 intercrosses (Bachmanov et al. 2002; Belknap and Atkins 2001). If silencing modifier loci are relatively common, it will be necessary to modify the MCM strategy such that a sufficient number of animals are genotyped genome-wide to detect the expected interactions. Three, we need to consider that using a balanced panel of F_2 intercrosses may not be the most efficient MCM strategy for the detection of similar QTLs. Assuming that one would initiate a MCM project with some existing QTL information in hand, it may well be a more productive approach to vary only one of the mapping strains in an attempt to maximize the likelihood that a similar QTL will be detected. This approach, of course, assumes that there is a greater value associated with QTL detected as opposed to QTL not detected.

Although one goal of MCM is to improve QTL resolution (see above), there are obviously other strategies that accomplish this goal much more efficiently, e.g., mapping in advanced intercross animals (Darvasi 1998). However, if the primary goal of MCM is to estimate the haplotype structure of the QTL, are there satisfactory and more efficient MCM alternatives? At the time the current studies were initiated (Spring 2000), the most obvious alternative was mapping in HS animals. The advantages of this approach are several: (1) only a single mapping population is needed, (2) if the HS population is at an advanced generation, QTL precision is greatly enhanced (Talbot et al. 1999; Mott et al. 2000; Demarest et al. 2001; Hitzemann et al. 2002, 2003), and (3) theoretically, one can extract a complex signature for each QTL. The most widely cited disadvantage to this approach may be summarized as follows: Assuming that the intent of using the HS strategy was to further characterize a QTL or QTLs previously detected in diallele crosses, in HS animals the QTL may be silenced by modifier loci (see above), and even if a QTL is located in the appropriate chromosomal location, it will be difficult to conclude that QTLs detected in an intercross and in HS animals are identical. Talbot et al. (1999) were the first to note such differences between mapping QTLs for open-field response in a B6 × C F_2 intercross and in HS animals formed by crossing eight laboratory strains, including the B6 and C strains. Mott et al. (2000) provided a solution to this problem by developing a mapping algorithm that took into account the complex haplotype structure of the HS animals; not only was mapping resolution improved, the algorithm estimated the contribution of each of the eight strains to the QTL. Based on the estimated QTL position and on the estimated contribution of the B6 and C strains, the majority of the QTLs de-

tected in the B6 × C intercross were found in the HS animals. The problem with this analysis strategy was that the confidence intervals associated with each strain effect were quite broad and, thus, the true haplotype signature was not precisely known. This problem, of course, is related to the difficulties associated with having sufficient data to accurately determine which of 36 possible genotypes are present at any given locus. Arguably, we now recognize that the actual number of different genotypes may well be far less than 36 (Wade et al. 2002; Wiltshire et al. 2003; Cervino et al. 2005), but nonetheless the problem remains complex. In response to this problem, the decision was made to develop a new HS population derived from the four MCM progenitor strains. For such a cross there are only ten potential genotypes, and, thus, there should be far fewer problems (compared with the eight-way cross) in accurately determining the genotype. We recognized that the QTL signature that would be extracted from a four-way cross would have much less information content than the signature extracted from a eight-way cross but it was likely to be more accurate and would still be a significant improvement over the haplotype information extracted from an F_2 intercross or a backcross.

The HS4 animals were interrogated at G_5 for some of the QTLs detected in the six F_2 intercrosses. Data are presented for the QTLs on Chrs 1 and 2 which suggest that very similar QTLs to those found in the intercrosses are found in the HS4 animals. Importantly, it should be noted that for Chr 1, the QTL was detected for the B6:C-D2-LP contrast but not the B6-C:D2-LP contrast. These data suggest that the weak QTL found in the B6 × C intercross is probably real and that the haplotype structure of the QTL likely can be characterized because the B6 allele is different in the same way from the C, D2, and LP alleles (and the alleles from these strains are likely to be identical at the QTL). As noted previously (Hitzemann et al. 2003), there appear to be several domains on distal Chr 1 that meet these criteria. In this regard it is of interest to note that the HS4 data (Fig. 7) suggest that there may well be two QTLs present on distal Chr 1. The QTLs on Chrs 8, 9, and 13 were also examined (data not shown); the QTLs on Chrs 8 and 13 but not 9 were confirmed and appeared to have the correct haplotype structure. Thus, our data confirm and extend the conclusions reached by Mott et al.(2000) regarding mapping known QTLs in HS populations (see above).

The QTL on Chr 2 was reexamined in the HS4 animals at G_{19}. The G_5 data were unclear as to the haplotype structure of the QTL, i.e., essentially identical information was obtained from the B6:C-D2-LP and B6-LP:C-D2 contrasts. As noted in Fig. 9, the region of interest on Chr 2 is largely composed of alternating regions of B6:C-D2-LP and B6-LP:C-D2 blocks. The QTL in the HS4 animals was clearly associated with the B6-LP:C-D2 block located between 112.5 and 117.5 Mbp and is clearly absent from the B6:C-D2-LP blocks. One interpretation of these data is that our search for the quantitative trait gene or genes should focus on sequence and/or expression variants with the B6-LP:C-D2 haplotype. However, this conclusion should be viewed cautiously. We again note that the Chr 2 QTL was not detected in the D2 × LP, C × B6, or C × LP intercrosses; while the failure to detect a QTL may have resulted from the effects of modifying loci (see above), there are other explanations. Consider the following: Although the pervasive allelic pattern in the block containing the QTL is clearly B6-LP:C-D2, Yalcin et al. (2004) have observed that within such blocks there is considerable allelic microheterogeneity which in turn is likely to require complete sequence information to successfully map the QTL. Thus, from the sort of data presented in the current study, we know the characteristics of the domain that contains the QTL but we may or may not know the QTL's specific characteristics. Despite this caveat, mapping in HS animals retains two significant advantages over MCM: (1) only a single population of animals needs to be phenotyped and genotyped, and (2) the mapping precision generally exceeds what can be obtained with MCM of similar overall sample size. Allelic microheterogeneity is retained as a problem for all methods: MCM, multiple-strain mapping, and mapping in advanced generations of HS animals.

In conclusion, the duration of the current study coincided with a period that witnessed some remarkable advances in strategies for detecting and fine mapping QTLs. However, the goal of developing a high-throughput strategy for moving from QTL to QTG and finally to the quantitative trait nucleotide remains elusive. Resources needed to meet this goal will include detailed multistrain sequence information and gene expression data from multiple tissues, and for behavioral QTLs, gene expression data from multiple brain regions and cell types. The development of these resources will need to be a community-wide effort. To be most efficient, this effort will require some agreement to use standardized mapping populations. Here, we simply note that the HS4 animals described here and HS-NPT animals developed previously (Demarest et al. 2001) are freely available. The latter were recently used in a very large QTL experiment (n = 2000) which focused on

multiple physiologic and behavioral phenotypes (Solberg et al. 2006).

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