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A major influence of sex-specific loci on alcohol preference in C57Bl/6 and DBA/2 inbred mice

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Abstract. C57Bl/6 mice reproducibly prefer to ingest more 10% ethanol in a two-bottle choice paradigm than do DBA/2J mice. In this paper we report the identification of two new sex-specific alcohol preference (*Alcp*) loci. Melo and associates (1996) identified two loci: *Alcp1*, a male-specific locus on Chromosome (Chr) 2, and *Alcp2*, a female- and cross-specific locus on Chr 11. We have additionally identified *Alcp3*, a male-specific locus on Chr 3, and *Alcp4*, a female-specific locus on Chr 1. We have also performed a statistical analysis to exclude the possibility of undiscovered major alcohol preference loci that are not sex-specific in our backcross paradigm. Our results indicate that alcohol preference in C57BL/6 mice, as measured in our backcross, is largely controlled in a sex-specific manner.

Introduction

Despite substantial efforts over nearly a century, the causes that underlie human alcoholism are relatively poorly understood. It is generally agreed that there is a genetic component to human alcoholism, but until recently there has been relatively little progress in determining the exact nature of that component (Devor and Cloninger 1989; Begleiter and Kissin 1995). Recently, however, the Collaborative Study on the Genetics of Alcoholism (COGA) has made significant strides. Using a number of genetic strategies in a large sample population of alcoholics, COGA was able to show suggestive linkage of alcoholism-related phenotypes to human Chrs 1, 2, 4, and 7 (Reich et al. 1998). COGA was also able to examine phenotypic markers for alcoholism (Porjesz et al. 1998) and provide more evidence against involvement of the dopamine D2 receptor (DRD2), a much discussed potential marker for alcoholism (Edenberg et al. 1998). It is likely that many of these suggestive loci represent genuine linkage to genes related to alcoholism in humans.

Difficulties arise in human studies from the fact that alcoholism is a complex disorder. Multiple levels of social influence combine with genetic heterogeneity and the polygenic nature of the trait to create a scenario that is at best difficult to unravel. Many of the difficulties inherent in the human system can be overcome with the use of experimental animals as model systems. Animal models have the great advantage of allowing informative matings to be performed. Studies of traits associated with alcohol consumption have proliferated in a number of animal systems, including Drosophila, mice, rats, and monkeys. Of course, it is quite unlikely that any one animal system will be an adequate model for human alcoholism. Although this is a good reason to treat animal data with some caution, it by no means invalidates the use of animal systems in investigating alcoholism. Indeed, for the reasons noted above, even a human subpopulation is unlikely to embody all of the characteristics we associate with alcoholism, and considerable effort has been expended in subdividing alcoholic populations into groups that only evidence a subset of the traits commonly attributed to this disease.

One of the more widely studied rodent models is the C57BL/6 (B6)–DBA/2 (D2) system. McClearn and Rodgers (1959) originally described the high-alcohol preference phenotype of B6 animals and the extreme alcohol avoidance phenotype of D2 animals in a paper surveying alcohol preference variations in a number of inbred mouse strains. It is clear that, even in mice, alcohol response is a complex issue. For instance, while B6 mice show higher alcohol preference, D2 mice show a greater increase in locomotor activity after ethanol exposure than do B6 mice (Cunningham et al. 1992; Dudek et al. 1991; Tabakoff and Kiianmaa 1982), as well as worse handling-induced convulsions (Goldstein and Kakihana 1974).

While these unusual phenotypes have been examined for years by pharmacological approaches, neurobiological techniques, and biochemical analyses, only in recent years has it become possible to dissect the genetic components of the phenotypes. Early efforts focused mainly on the use of B6xD2 recombinant inbred (BXD RI) strains generated by repeated inbreeding (for at least 20 generations) between F_2 animals whose ultimate progenitors were the B6 and DBA strains. Using RI lines, Rodriguez et al. (1994) demonstrated that there was moderate heritability of alcohol preference. They were also able to derive correlations between alcohol preference, alcohol acceptance, and hypnotic dose sensitivity (HDS). Their results suggested that preference and acceptance are only modestly correlated, while HDS was not correlated with either measure. Because it is likely that preference, acceptance, and HDS have substantially different genetic underpinnings, we will address only results of studies using substantially similar protocols when considering the consistency of mapping results.

Rodriguez and coworkers (1995) later reported the results of a mapping study with BXD RI lines. In this study they reported linkage of alcohol preference to Chrs 1, 2, 6, 7, 10, 11, 15, and 17. Using a larger marker set, Tarantino and colleagues (1998) reanalyzed the Rodriguez et al. (1995) data and additionally identified putative QTLs on Chrs 4, 5, 8, and 18. Additionally, Phillips and associates (1994) identified putative QTLs for 10% ethanol preference on Chrs 2, 3, 4, 7, and 9. Unfortunately, RI analyses are subject to the sample size limit imposed by the number of available lines and seldom reach the significance level later recommended by Lander and Kruglyak (1995). For this reason, Tarantino et al. (1998) also undertook an analysis of an F_2 population to confirm QTLs nominated in their RI work and identify new QTLs for ethanol preference. Using this protocol, they identified three significant loci on Chrs 1, 4, and 9 as well as three suggestive loci on Chrs 2, 3, and 10.

Melo and coworkers (1996) also chose to study the simplest of the B6/D2 alcohol-related phenotypes, alcohol preference, in a

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two-bottle choice paradigm. This paper confirms and extends many of the results obtained in that original study with the completion of the genome scan initiated by Melo et al. (1996), confirmation of some loci, and examination of the issue of sex specificity for this trait in our paradigm.

Melo and associates used (B6 × D2) × B6 and B6 × (B6 × D2) backcross breeding protocols to generate a segregating population which they then used to map two alcohol preference loci. *Alcp1* mapped to Chr 2, and *Alcp2* mapped to Chr 11, at significance levels exceeding the threshold suggested by Lander and Kruglyak (1995). Surprisingly, both of these loci are sex specific. *Alcp1* is a locus with a male-specific effect residing on Chr 2. *Alcp2* is, if anything, more unusual. It apparently is a locus with both sex and cross specificity, since the parental origin of this particular segment of Chr 11 seems to have an effect only on females with B6 fathers and F_1 mothers.

The differences in the distributions of alcohol preference for B6 males and females (Melo et al. 1996), as well as the differences in distributions for males and females in our backcrosses, suggest that there are some non-overlapping sex-specific genetic influences involved in this trait. Rodriguez et al. (1994) noted the same phenomenon in their RI lines, as did Tarantino et al. (1998) in their intercross. It is reasonable to hypothesize that there are loci with some effect in one sex and a larger or smaller effect in the other sex. Alternatively, it is possible that a minority of the preference phenotype derives from sex-specific loci, while the majority of the preference phenotype derives from non-sex-specific loci. The lack of non-sex-specific loci in our backcross, however, raised the possibility that neither alternative reflected the actual situation.

Materials and methods

Animals. C57BL/6J (B6), DBA (D2), and B6 × D2 (F_1) mice were purchased from The Jackson laboratory or bred at Princeton University. F_1 × B6 and B6 × F_1 (collectively referred to as N_2) animals were bred at Princeton University. N_2 animals used to generate the first set of experimental data (referred to here as the first set; the set used to detect *Alcp1* and *Alcp2*) were phenotyped at between 2 and 9 months of age, as described below. Animals used to generate the second set of experimental data (referred to here as the second set; created to confirm the suggestive loci described below) were phenotyped at between 3 and 5 months of age.

Phenotyping. Alcohol preference was measured in a two-bottle choice test. Two identical bottles, one filled with 10% (wt/vol) ethanol solution and the other with water, were presented in a 24-h unlimited access paradigm with ad libitum food. Bottles consisted of Corex or plastic tubes topped by #3 stoppers with 2-inch ball-less sipper tubes. At the end of each trial, old bottles were removed and replaced with a freshly prepared set. In an effort to control for possible position preference, the positions of the ethanol and water bottles were reversed after each trial, although no position preference was noted.

Preference was recorded as the percentage of ethanol consumed divided by the total fluid consumed over a 3-day trial. Three trials per mouse were conducted for the first set and four trials per mouse were conducted for the second set. In the second set, the average evaporation from these types of tubes was measured and subtracted from the raw scores.

A test for consistency of measures was also applied to the preference scores obtained for each animal. When all values for a given animal were within 2 sex-specific B6 standard deviations of each other, they were averaged together to determine the preference score. The value for a B6 sex-specific standard deviation was determined by the preference scores for a population of B6 animals as described elsewhere (Melo et al. 1996) and was a difference of 0.10 in preference score for females and 0.16 in preference score for males.

When this was not the case, only the two (first set) or three (second set) closest values were retained. If this put all values within 2 sex-specific B6 standard deviations, the average was taken. Otherwise, the animal was removed from consideration. This was necessary only for 3 of 338 N_2s in the first set and 2 of 160 N_2s in the second set. The consistency standard was applied to all animals before any genotypic data were correlated.

Genotypic analysis. Genomic DNA was prepared from tissues according to standard protocols. Primers for microsatellite markers polymorphic between B6 and D2 (Dietrich et al. 1994) were purchased from Research Genetics (Huntsville, AL) or synthesized in-house, and PCR was performed as suggested by Research Genetics. Microsatellite markers were typed by electrophoresis in 6% acrylamide or 3% FMC (Rockland, ME) Metaphor agarose and stained with ethidium bromide. Markers with exceptionally small polymorphisms were detected by autoradiography. Since the genetic protocol used was a backcross to B6, animals could be either heterozygous B6/D2 or homozygous B6/B6.

Data analysis. All data entry, storage, and analyses were performed with Microsoft Excel 98 on a Power Macintosh 7200/120 (Apple). T-tests were performed with Excel add-ins with correction for unequal variances (also referred to as Welch's *t*-test or the separate variance *t*-test). Chi-squared tests were performed by hand or by evaluating the appropriate formula within Excel. Stepwise regressions were performed with the Excel add-ins regression function.

Linkage analysis. The linkage analysis presented is the completion of the genome scan initiated by Melo and associates (1996). The initial genome scan was completed in the set of animals phenotyped and partially genotyped by Melo and coworkers, referred to here as the first set. Confirmation of loci was carried out with a separate set of animals phenotyped for that purpose, referred to as the second set.

In the genome scan performed on the first set of animals, 110 microsatellite markers (Dietrich et al. 1994) were chosen approximately every 15 cM and genotyped as described for the 20 males and 20 females in the set that expressed the highest levels of alcohol preference. Analysis of data was performed using a Chi-squared analysis for high-preference animals. Loci in high-preference animals with greater than 60% B6/B6 alleles were investigated further by genotyping the next 20 highest animals. If the marker still showed greater than 60% B6/B6, the entire population was genotyped at that marker.

It was during this initial analysis of animals in the genome scan that the markers *3-200* and *1-295* were identified as having potential linkage to alcohol preference QTLs. (Here and henceforth, we will abbreviate all microsatellite markers by removing the initial D and internal Mit designations.) As with *Alcp1* and *Alcp2*, all N_2 animals of the appropriate sex in the first set were genotyped at these markers, and the preference scores of animals homozygous B6/B6 at the markers were compared with the scores of animals heterozygous at the locus. However, while this effort yielded suggestive results, it did not generate a *p*-value considered significant with the threshold criteria of Lander and Kruglyak (1995). We therefore generated an additional set of N_2 animals as described above and genotyped all N_2s of the appropriate sex to attempt to confirm these suggestive markers. Additionally, we genotyped this second set of N_2 animals at the locations of *Alcp1* and *Alcp2* in an attempt to confirm these loci.

Analysis of locus interaction. Since there are now two alcohol preference loci identified for each sex, it was possible to determine whether the two loci are involved in overlapping pathways. We examined epistatic, synthetic, complementary and additive models, using data from the first set of animals. In each case, the animals that were homozygous and heterozygous at each of the loci operating in that sex were separated, making four categories. Only animals genotyped at markers across both intervals were considered. (For *Alcp2* and *Alcp4*, only females with B6 fathers were analyzed.) The double-homozygote category consisted of the animals that were B6/B6 at both loci. The first single homozygote category consisted of animals that were B6/B6 at the first locus and B6/D2 at the second locus (and likewise for the second single homozygote). The double heterozygote consisted of those remaining animals that were B6/DBA at both loci.

For an epistatic interaction, homozygosity at one locus would remove the effect of differing alleles at the second locus. In the case of the first locus being epistatic to the second locus, the double homozygote and the first single homozygote should have the same phenotype. If, on the other hand, the second locus is epistatic to the first locus, the situation should be reversed. We tested for an epistatic interaction by performing Welch's t-tests between the double and single homozygous states for the first and second locus respectively. A significant result indicated a lack of epistasis.

For a synthetic interaction, homozygosity at both loci would generate a high-preference phenotype, and all other combinations of homozygosity and heterozygosity would give a low-preference phenotype. For a comple-

mentary interaction, homozygosity at either locus or both loci would give approximately the same preference phenotype, whereas heterozygosity at both loci would give a lower preference phenotype. We tested for these types of interactions using an ANOVA. For a synthetic interaction, the ANOVA included the double heterozygote and both single homozygotes, which should all give the same phenotype under this model. For a complementary interaction, the ANOVA included the double homozygote and both single homozygotes, which should also all give the same phenotype under this model. A significant result indicated a lack of synthetic or complementary interactions respectively.

The last alternative is that there is no interaction between the loci. Under this additive model, the effect of one locus is unrelated to the effect of another. Our examination of this model was somewhat more descriptive. We first examined the difference between the average scores in the double homozygote and the first single homozygote versus the difference in average scores between the second single homozygote and the double heterozygote. We next examined the analogous differences between the double homozygote and second single homozygote and between the first single homozygote and the double heterozygote. Under an additive model, these differences should be equal.

We also performed a stepwise regression, first adding the first locus on the first step and the second locus on the second step. We noted the coefficients for each step and then reversed the stepwise order of addition. Under an additive model, it should not make any difference in which order the loci are included. (In a synthetic model, on the other hand, neither locus individually would have a notable contribution, and in a complementary model, the first locus added should have a contribution while the second should have none in either order of addition. The stepwise regression thus serves as a check on tests of these models as well.)

Non-sex-specific locus exclusion analysis. Exclusion of non-sex-specific loci was performed with a modified chi-squared procedure. Ordinarily, when using a chi-squared test, the expectation is that there is no linkage (null hypothesis), and the experimenter is looking for linkage (alternate hypothesis) at some level of confidence. In this case, linkage at a level similar to or greater than the level shown with *Alcp1* and *Alcp2* was the null hypothesis, and no linkage or linkage at a level less than that observed for *Alcp1* and *Alcp2* is the alternate hypothesis. In other words, the presence of non-sex specific loci with an effect size similar to *Alcp1* and *Alcp2* should cause us to accept the null hypothesis (linkage) and fail to exclude the area.

For this test, the highest alcohol-preferring 31 females and top 23 males from the first set and the top 10 females and 5 males from the second set were combined. These animals represent the top 54 of 336 phenotypes in the first set and the top 15 of 160 phenotypes in the second set. Because the animals from each set were combined, we had a total of up to 69 animals for each marker analyzed. (It was not necessary to collect marker information for all 69 animals in each case. If the region could be excluded with fewer data points, we did not necessarily complete the remaining genotypes.) The null hypothesis was that 70% of the highest preferring animals would be homozygous (B6/B6). This level of association was chosen because it is slightly lower than the strength of linkage found for the first two *Alcp* loci. (For the 40 animals used in the first genome scan, *Alcp1* has a male-specific association of 75%, and *Alcp2* has a female- and cross-specific association of 92%. Of the loci that will be discussed in this paper, *Alcp3* has a male-specific association of 75%, and *Alcp4,* the weakest locus, has a female-specific association of 65%).

A chi-squared test was performed for each of the markers in the genome scan with an expected value of 70% B6/B6 homozygotes as discussed above, and a criterion of $p < 0.01$ for significance. Often it was unnecessary to have marker information for all of the animals, and further characterization was not attempted after significance was established. For the markers that had previously been identified as being associated with a known sex-specific QTL, only those animals of the opposite sex and/or cross were used to determine the absence of a similarly strong non-sex-specific QTL.

For markers that were impossible to exclude with these criteria, marker information across the entire population was established, and a similar scheme was imposed. First, the data were corrected by subtracting out the effect of a presumptive locus. This was done by subtracting 0.11 from the preference scores of homozygous animals. (For all loci, the difference between the mean of homozygous and heterozygous animals preference scores was at least 0.11). A T-test was then performed on the modified data set. In this case, the null hypothesis was a difference of at least 0.11 between the preference scores of heterozygotes and homozygotes (that is, a locus), and the alternate hypothesis was absence of a locus at that point or presence of a locus significantly weaker than *Alcp1* or *Alcp2.*

Table 1. Average preference scores for the first and second sets of N_2 animals, grouped by sex and cross.[a]

Cross Type	Both Sexes	Males	Females	Significance
The first set				
Both Crosses	.43 ± .25 (335)	.38 ± .24 (184)	.50 ± .26 (151)	$p = 0.00004$
(B6 × D2) × B6	.43 ± .26 (262)	.38 ± .24 (147)	.49 ± .27 (115)	$p = 0.0008$
B6 × (B6 × D2)	.46 ± .24 (73)	.39 ± .23 (37)	.53 ± .24 (36)	$p = 0.017$
Significance:	Not significant	Not significant	Not significant	
The second set				
Both Crosses	.31 ± .24 (160)	.26 ± .21 (90)	.37 ± .26 (70)	$p = 0.0017$
(B6 × D2) × B6	.31 ± .24 (82)	.24 ± .18 (47)	.40 ± .27 (35)	$p = 0.0012$
B6 × (B6 × D2)	.30 ± .24 (78)	.28 ± .23 (43)	.34 ± .25 (35)	$p = 0.1490$
Significance:	Not significant	Not significant	Not significant	

[a] Preference scores are reported as average ± standard deviation. The number of observations is in parentheses.

Results

Distribution of alcohol preference scores in the first and second data sets. As previously mentioned, the alcohol preference scores differed between the first and second data sets, probably as a result of the modifications in protocol mentioned in the Phenotyping section of Materials and methods. As shown in Table 1, the averages for the second set of animals were largely parallel, but somewhat lower. This is also reflected in the distribution shown in Fig. 1. The distribution for the second set of animals is otherwise similar to the first. As has been previously found, we note that male and female averages and distributions are generally significantly different.

Identification of two new sex-specific loci. Completion of the genome scan begun by Melo and colleagues (1996) yielded a number of potential loci, which were further investigated by typing the entire population of the first set for nearby markers and performing sex-specific *t*-tests between animals that were homozygous B6 and heterozygous B6/D2 at the marker. In most cases, the investigated loci failed to show an effect in the whole population and were considered false positives. However, markers 3-200, 1-295, and others nearby continued to show suggestive evidence of linkage in the whole population analysis. As Table 2 shows, both of these markers had *p*-values exceeding the criterion ($p = 0.0034$) advanced by Lander and Kruglyak (1995) for a suggestive locus in one sex but not the other.

In order to determine the status of these suggestive loci, we bred, phenotyped, and genotyped the second set of N_2 animals as described in the Materials and methods. Genotyping revealed that the regions considered suggestive in the first set of animals were also significantly ($p < 0.05$) associated with differences in alcohol preference in the second set of animals. Further, the sex specificity observed in the first set was maintained in both cases. This suggestive association, followed by confirmation in a second data set, demonstrates that we have identified two new, significant loci.

Unfortunately, it is not straightforward to combine the data from our two crosses to construct a confidence interval for these new loci. We chose, therefore, to report an interval based solely on genotyping of the first set of animals. Intervals for these new loci are shown in Figs. 2 and 3.

Examination of previously identified ALCP loci. Since it was necessary to generate a second set of N_2 animals to confirm the presence of *Alcp3* and *Alcp4,* we were also able to examine the loci previously identified by Melo and coworkers in a second data set.

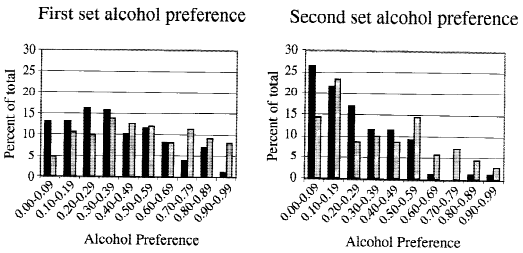

Fig. 1. Frequencies of alcohol preference for the first and second set of animals were determined and plotted. Black bars are male preference frequencies expressed as a percentage of total animals, and stippled bars are female preference frequencies expressed as a percentage of total animals.

Table 2. Sex-specific *p*-values for *Alcp* loci for first and second sets of animals.

		First Set		Second Set	
Alcp	Marker	Males	Females	Males	Females
Alcp1	*2-241*	0.000009[a]	0.33	0.06[a]	0.25
Alcp2[b]	*11-195*	0.38	0.00004[a]	0.44	0.14
Alcp3	*1-295*	0.34	0.002[a]	0.39	0.04[a]
Alcp4	*3-200*	0.003[a]	0.97	0.05[a]	0.29

[a] Significant ($p < .0001$ in the first set and $p < 0.05$ in the second set) or suggestive ($p < .0034$ in the first set and $p < .06$ in the second set) *p*-values. The *p*-values for the first set were previously published by Melo et al. (1996).
[b] For *Alcp2,* females refers only to females with B6 fathers, since *Alcp2* is cross-specific as well as sex-specific.

Our second experimental data set was analyzed at markers associated with the loci *Alcp1* and the *Alcp2* identified in the first data set by Melo and associates (Table 2). $P < 0.06$ was obtained for linkage with marker *2-241* in females. This result provides further support for the existence of the *Alcp1* locus and its sex-specific effect on alcohol preference in the backcross paradigm.

Unfortunately, we were unable to confirm linkage to *Alcp2,* the second locus identified by Melo and colleagues. Our inability to confirm *Alcp2* was disappointing but not necessarily unexpected. *Alcp2* was detected in the first place only because 76% of the 151 females analyzed in the first set were of the correct cross (115 animals). In contrast, only 50% of the 70 females analyzed in the second set were of the correct cross (35 animals). This much smaller number of animals greatly decreases the power to detect the effect of the locus. Since the major purpose of generating a second set of animals was the confirmation of the two novel loci, however, and not the confirmation of the previous loci, we did not generate the additional females that would have been necessary to confirm *Alcp2.*

Interactions. We examined our data for interactions between the sex-specific loci identified so far. We looked for epistatic, synthetic, and complementary interactions for each pair of loci, using the testing procedures outlined in the Methods section.

For *Alcp1* and *Alcp3,* we were able to eliminate the likelihood of epistatic interactions in either direction ($p = 0.05$ for *Alcp1* epistatic to *Alcp3* and $p = 0.007$ for epistasis in the other direction). Additionally, we were able to conclude that our data are inconsistent with synthetic ($p = 0.02$) and complementary ($p = 0.04$) interactions. Stepwise regression was consistent with an additive model, but not with a synthetic or complementary model. Coefficients for *Alcp1* of 0.14 and 0.13 and coefficients for *Alcp3* of 0.10 and 0.09 were observed when those loci were added on the first and second steps respectively.

For *Alcp2* and *Alcp4,* we were also able to eliminate epistatic interactions in both directions ($p = 0.02$ for *Alcp2* epistatic to *Alcp4* and $p = 0.03$ for epistasis in the other direction). Our data for these loci were also relatively inconsistent with synthetic ($p = 0.07$) and inconsistent with complementary ($p = 0.01$) interactions. Stepwise regression was consistent with an additive but not a synthetic or complementary model. Coefficients for *Alcp2* of 0.19 and 0.18 and for *Alcp4* of 0.16 and 0.14 were observed when these loci were added in the first and second steps respectively.

Qualitatively, as can be seen in Table 3, however, both sets of sex-specific loci seem to be additive with respect to each other. Comparison of differences across the several homozygous and heterozygous conditions as described in the Methods section, as well as the results presented above, suggests that there is no interaction between the loci identified so far. On the contrary, our results are consistent with expectations for two completely additive loci of approximately equal strength in both cases.

Exclusion of non-sex-specific loci on autosomes by modified chi-squared analysis. Using the statistical procedure described in Materials and methods, we were able to exclude ($p < 0.01$) non-sex-specific loci that would have resulted in the same or a larger number of B/B homozygotes (70%) at the high end of the phenotypic distribution for much of the genome. In fact, with the exception of the proximal end of the X Chr (0–52 cM), the entire genome was successfully excluded at this level of significance. At a traditional significance level of $p < 0.05$, it was further possible to exclude all but 0–20cM of the X Chr.

The inability to exclude the proximal X Chr could have resulted from a non-sex-specific (or sex-specific) locus in the area or could have been a spurious result. In order to determine which was the case, we performed a modified T-test, as described in Materials and methods.

Exclusion of non-sex-specific loci on the proximal X Chr. Data for a larger sample of first series animals were taken and analyzed, as described for two markers on the proximal end of the X Chr. The modified T-test allows us to exclude linkage of *X-124* (17 cM; $p = 0.01$) and *X-089* (2 cM; $p = 0.05$). Since these two markers flank the region that could not be excluded, we are able to exclude the possibility of non-sex-specific loci (similar in strength to the sex-specific loci already uncovered) on the proximal end of the X Chr.

Discussion

In a previous report, we presented evidence for the existence of two loci—*Alcp1* and *Alcp2*—that play major roles in the alcohol preference phenotype of B6 animals. Both loci were found to act in a sex-specific manner—*Alcp1* acts only in males, and *Alcp2* acts only in females. In this report, we provide further independent evidence in support of the sex-specific action of *Alcp1* and describe the identification of two additional alcohol preference loci—*Alcp3* and *Alcp4*—that also act in a sex-specific manner.

Relation to previous work. It is notable that suggestive loci in similar regions have been identified near *Alcp1* and *Alcp3,* with an intercross breeding protocol and a somewhat different preference paradigm (Tarantino et al. 1998). Tarantino and coworkers also identified a significant locus on Chr 1 that overlaps *Alcp4,* but whose 1 LOD support interval is distal to that of *Alcp4.* Using short-term selected lines, Belknap and associates (1997) also identified a suggestive alcohol preference locus near *Alcp3* and a more modestly suggestive locus near *Alcp1.*

Additionally, RI studies have previously nominated QTLs in similar regions. Rodriguez and colleagues (1995) reported a potential QTL on proximal Chr 1, linked to *D1MIT5* (33 cM), as well as a potential QTL on Chr 2, linked to *Mdk* (53 cM). It is interesting to note that each of these two QTLs had a significance of $p < 0.001$, well within the Lander and Kruglyak (1995) criteria for a suggestive locus of $p \leqslant .0034$ and rather impressive for an RI study of a complex trait. Rodriguez and coworkers (1995) also

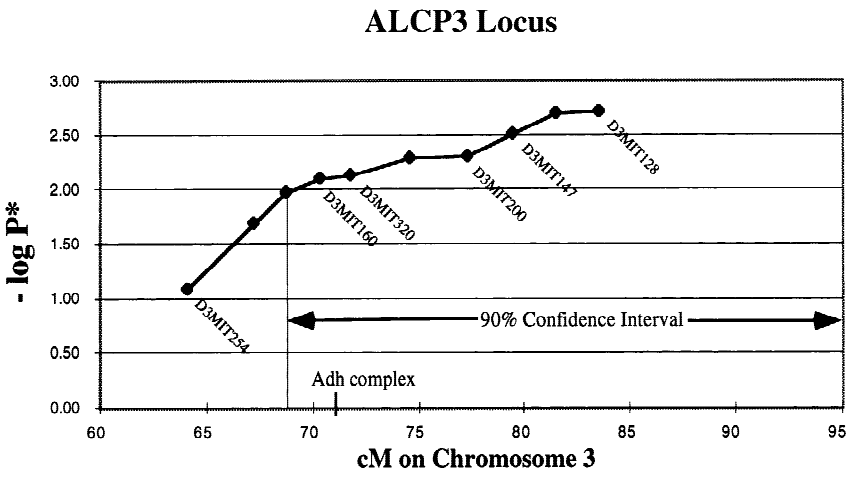

Fig. 2. *Alcp3* is a male-specific locus on Chr 3. The confidence interval extends to the end of the chromosome owing to a lack of MIT markers with appropriate polymorphisms. Adh is the alcohol dehydrogenase complex that is involved in ethanol metabolism.
*It is important to note that the -logP value given is from the first set of animals only and is used only to estimate the confidence interval for the locus. The presence of both novel loci was confirmed with a second set of animals, as described.

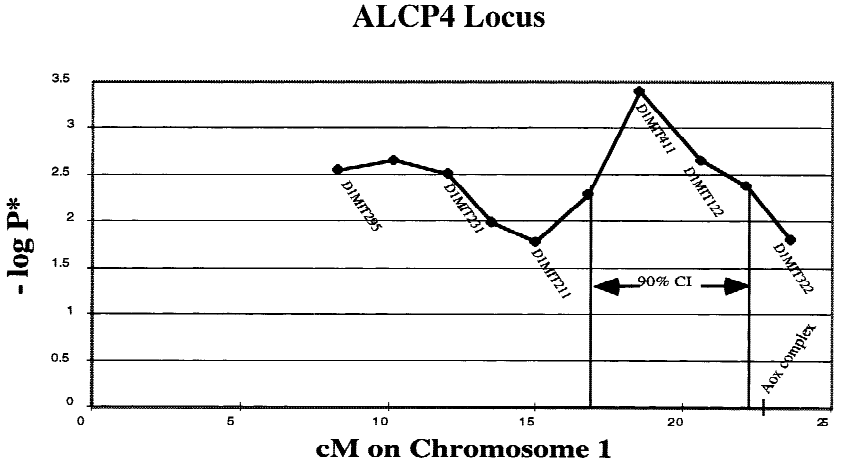

Fig. 3. *Alcp4* is a female-specific locus on Chr 1. Aox is the aldehyde oxidase complex, which is believed to be involved in ethanol metabolism.
*It is important to note that the -logP value given is from the first set of animals only and is used only to estimate the confidence interval for the locus. The presence of both novel loci was confirmed with a second set of animals, as described.

Table 3. Modes of action of *Alcp* loci.[a]

Male-specific loci	*Alcp1* B/B	*Alcp1* B/D	*Alcp1* Delta
Alcp3 B/B	0.50 (.05) n = 45	0.36 (.06) n = 42	0.14
Alcp3 B/D	0.40 (.05) n = 32	0.24 (.03) n = 32	0.16
Alcp3 Delta	0.10	0.12	
Female-specific loci	*Alcp2* B/B (B6 father)	*Alcp2* B/D (B6 father)	*Alcp2* Delta
Alcp4 B/B	0.61 (.07) n = 36	0.41 (.05) n = 32	0.20
Alcp4 B/D	0.50 (.07) n = 15	0.31 (.05) n = 24	0.19
Alcp4 Delta	0.11	0.10	

[a] As discussed in the text, this table shows each locus broken down by genotype across the interval. The values given in the table are alcohol preference scores taken from the first data set and are presented as preference (standard error) n = sample size. The delta in each case represents the difference between the average scores for animals that are heterozygous (B/D) and homozygous B6 (B/B) across each interval. If all deltas are the same, the results are consistent with a completely additive model. In other words, the genotype at the second locus is irrelevant to the effect of the locus being examined.

noted another possible QTL linked to *Mpmv22* on Chr 1 (107 cM), which could explain the extremely wide confidence interval reported by Tarantino and associates (1998) if they are detecting both *Alcp4* and a novel locus near the distal end of Chr 1.

Note should also be taken of the loci that were reported in Tarantino and coworkers (1998) and not in this study or by Melo and colleagues (1996), and vice versa. The easiest case to consider is that of *Alcp2*. The cross specificity of this locus convincingly explains why Tarantino and other workers have not previously detected a locus in this region. Since this locus acts in our backcross as if it were paternally imprinted, it would be impossible in an F_2 animal heterozygous at this locus to predict whether the animal should have a higher or lower alcohol preference score. The marker *D11Nds2* (62 cM) was, however, nominated by Rodriguez et al. (1995) in their RI study, though not confirmed by Tarantino et al. (1998).

Tarantino and colleagues (1998) also report significant loci on Chrs 4 [nominated in Tarantino's reanalysis of the data from Rodriguez et al. (1995)] and 9 [nominated by Phillips et al. (1994)], as well as another suggestive locus on Chr 10 [nominated by Rodriguez et al. (1994)].

The locus on Chr 10 has a maximum peak of LOD 2.0 in Tarantino's alcohol preference paradigm, however, so we do not consider it in the analysis below. It is, however, interesting to note that for the locus on Chr 10 (both in Rodriguez et al. and in Tarantino et al.) the D2 allele is the increasing allele.

Much of the difference between our results and those of Tarantino and coworkers can be ascribed to differences in the nature of the genetic protocol employed. We use an intercross-backcross mapping protocol, which is optimal for detecting alleles that are D2 dominant/B6 recessive in action. Because one copy of the genome in N_2 animals always comes from the B6 parent, our protocol will not detect B6 dominant alleles at all and is likely to miss partially dominant B6 alleles. The QTLs detected by Tarantino on Chrs 1, 4, and 9 are most consistent with either B6 dominant (Chr 1) or additive and B6 dominant (Chrs 4, 9) models. In contrast, the suggestive loci on Chrs 2 and 3 are consistent with a B6 recessive model. The B6 dominant nature of the locus detected by Tarantino on Chr 1 suggests that this locus may in fact be

distinct from *Alcp4*. If this is the case, the respective results of these two approaches are entirely expected: We strongly detect recessive loci on Chrs 1, 2, and 3 and miss the additional dominant and partially dominant loci on Chrs 1, 4, and 9.

The sex specificity of our results, however, is an issue apart from detection. It may also result from differences in either the genetic or phenotypic paradigm. Alcp1 and Tarantino's locus on Chr 2 are likely to represent the same genetic entity, but Tarantino's results are not sex specific. Since we have confirmed the sex specificity as well as the presence of this locus in a separate set of animals, we must conclude that the difference is inherent in the paradigms examined.

For *Alcp3*, however, the results are somewhat more promising. We have confirmed the presence and sex specificity of this locus in two separate sets of animals. Additionally, Tarantino reports that their suggestive locus on Chr 3 has a maximum LOD of 4.5 in males and 0.6 in females. While addition of a second test prevented Tarantino and colleagues from reporting this as a significant locus, it seems likely that both the locus and the sex specificity are real and reproducible between our respective paradigms. However, Phillips and associates (1994) reported a locus in female mice at essentially the same position from RI data which they subsequently confirmed in short-term selected lines as a non-sex-specific locus (Belknap, 1997). Like the results of Tarantino's study and ours with respect to Chr 2, these seem to be replicable yet contradictory results for which we do not have an adequate explanation.

There do not seem to be any epistatic, synthetic, or complementary interactions between the loci identified, though because of the sex specificity we were able to examine only interactions between *Alcp1* and *Alcp3*, as well as those between *Alcp2* and *Alcp4*. In both sexes, the two relevant ALCP loci appear to be acting in an additive manner.

Our findings of four loci that affect alcohol preference in one sex but not the other, and of no loci that operate in both sexes, were surprising and unexpected. Our statistical analysis confirms the validity of this finding and leads us to conclude that alcohol preference in B6 mice is controlled predominantly in a sex-specific manner in our paradigm. It is important to note also that the limitation of our technique with respect to B6 dominant alleles also applies to the exclusion, and that the exclusion analysis will falsely eliminate increasing D2 alleles. The exclusion analysis does, however, allow us to eliminate the probability of non-sex-specific recessive B6 alleles that increase alcohol preference in our genetic and phenotypic system.

The identification of two new *Alcp* loci of strength similar to the original loci identified by Melo and coworkers means that we have explained an additional 27% of the genetic variance in males (*Alcp3*) and 28% in females (*Alcp4*). This means approximately 50% of the genetic variance in alcohol preference between B6 and D2 strains has so far been explained.

It is likely that we have identified the strongest *Alcp* loci in both males and females. It is possible that additional *Alcp* loci of near-equal strength are yet to be identified, but it is also possible that the unaccounted portion of the genetic variance is controlled by a large number of much weaker loci or by the B6 dominant loci described by Tarantino. It is not possible at this point to predict whether yet-to-be-identified Alcp loci will be sex-specific or not, though this report suggests that non-sex-specific loci in our paradigm are likely to be weaker than the sex-specific loci already identified.

Candidate genes for Alcp loci. We have not made any further progress towards identifying candidate genes in the *Alcp1* and *Alcp2* intervals, though *HTT* (the serotonin transporter) is still an intriguing candidate for *Alcp2*. Unfortunately, an initial sequencing of the HTT coding regions for the B6 and D2 strains did not uncover any strain-specific variations, suggesting that if HTT is responsible for the effect of *Alcp2*, the difference is likely to be a regulatory rather than a structural one.

Alcp3 has the most promising and likely candidate in its interval—the ADH (alcohol dehydrogenase) complex resides at 71.2 cM (Mouse Genome Database 1998) as noted on Fig. 2. *ADH* is clearly an important candidate because it is directly involved in ethanol metabolism. In humans, data from the COGA study suggest a possible protective locus on Chr 4, near the *ADH* locus (Reich et al. 1998). Additionally, alleles of *ADH* with higher activity are associated with lower levels of drinking in some Asian populations (Thomasson et al. 1991; Maezawa et al. 1995; Tanaka et al. 1996). However, it should also be noted that there is no demonstrated association between ADH alleles and protection from alcoholism in Caucasian or other non-Asian populations (Vidal et al. 1993; Gilder et al. 1993). Of course, since alcoholism is a complex disorder, the genetic causes of alcoholism may vary between populations. Since this is the case, it is not necessary that ADH be related to alcoholism in all populations to suppose that it may be acting in B6/DBA mice as it probably does in Asian populations.

Hepatic *ADH* activity has been reported to be higher in B6 mice than it is in D2 mice (Schlesinger et al. 1966, reviewed in Hunt 1996; Sheppard et al. 1968; Rao et al. 1997). Teichert-Kuliszewska (1988) found that in mice differing primarily in *ADH* alleles present, there was a minor difference in alcohol preference.

Unfortunately, the system of alcohol metabolism is not entirely straightforward, and various enzymes may play more or less important roles in different regions of the body. For instance, when B6 and DBA mice were intubated with an ethanol solution, DBA animals had a higher gastric alcohol dehydrogenase level and thus a lower blood alcohol level than did B6 mice (Desroches et al. 1995). Notably, there is precedent for the possible relation of alcohol metabolic enzymes to alcohol preference in mice. He and colleagues (1997) found an inverse relationship between ethanol preference and brain catalase activity, as did Gill et al. (1996). We take some caution from the fact that both He et al. and Gill et al. failed to find a straightforward relationship between polymorphic ALDH activity and ethanol preference, however.

Alcp4 also contains an intriguing metabolic candidate. The aldehyde oxidase complex maps to 23.2 cM on Chr 1. This complex is composed of two genes, *Aox1* and *Aox2*, which produce aldehyde oxidase. Aldehyde oxidase is involved in the regeneration of NAD+ from NADH (Gluecksohn-Waelsch et al. 1967), which is necessary for both steps of ethanol's metabolism to acetate. Aldehyde oxidase may thus play a role in facilitating alcohol metabolism. As with *Alcp3*, there is a known difference in aldehyde oxidase between the B6 and D2 strains. B6 mice have a tenfold higher level of aldehyde oxidase activity (Huff and Chaykin 1967). In addition to this intriguing known difference in activity, there is also evidence of hormonal control that could explain the sex specificity of *Alcp4* if the AOX complex is indeed responsible for the presence of the locus. In a reciprocal F_1 population, Huff and Chaykin (1967) further found that male F_1 animals had an AOX activity level near that of their B6 parents, whereas female F_1 animals had a level of activity intermediate between the parental phenotypes. Since there is little difference between the B6 parent and the male F_1, it is unlikely that there would be any larger difference in AOX activity between heterozygous and homozygous males in a backcross.

If higher aldehyde oxidase activity means less build-up of acetaldehyde, this result would be consistent with the hypothesis that human acetaldehyde levels after ethanol exposure are related to the expression of alcoholism (Thomasson et al. 1993). There is also precedent in rodents for a relationship between ethanol preference and aldehyde dehydrogenase concentrations. Alcohol-preferring mice and rats often have higher hepatic aldehyde dehydrogenase (ALDH) than their non-preferring counterparts (Koivisto and

Eriksson 1994; Koivula et al. 1975; Sheppard et al. 1970). It is, however, important to note that Gill et al. (1996) were unable to find a simple relationship between ALDH and alcohol preference, as were He et al. (1997). In fact, He et al. were unable to replicate the previously described differences in ALDH activity between the B6 and D2 strains.

Advanced mapping. Our identified *ALCP* loci are currently resolved to regions that are 15 to 20 cM across, which severely limits the reliability of the candidate gene analysis approach to dissecting the trait. We are currently exploring several possibilities, including Advanced Intercross Lines (AILs) to reduce the interval that must be examined for candidate genes. AILs are a multigeneration breeding protocol designed by Darvasi and Soller (1995) to enhance recombination frequency between two parental strains by means of an ''extended intercross.'' Our research in the immediate future will be directed towards narrowing the confidence intervals of our QTLs to less than 3 cM.

Conclusions

We have been able to identify two novel *ALCP* loci, which when combined with the loci detected by Melo et al. account for just under half of the high alcohol preference of the B6 strain. The most startling observation about this trait is that both male and female B6 animals have an extremely high preference for ethanol relative to other strains, but that the genetic factors underlying the trait seem to be largely different between sexes in our paradigm. Further work remains to be done to identify any remaining loci and to more precisely map, identify, and pursue candidates for the loci identified so far.

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