Confirmation and Fine Mapping of Ethanol Sensitivity Quantitative Trait Loci, and Candidate Gene Testing in the LXS Recombinant Inbred Mice

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ABSTRACT

In previous studies, we have mapped quantitative trait loci (QTLs) for hypnotic sensitivity to ethanol using a small recombinant inbred (RI) panel and a large F₂ backcross. Alcohol sensitivity is a major predictor of long-term risk for alcoholism. We remapped hypnotic sensitivity using a new set of 75 RI strains, the LXS, derived from Inbred Long Sleep and Inbred Short Sleep strains. We expected to improve mapping resolution in the QTL regions and to identify novel QTLs for loss of the righting reflex due to ethanol. We used three common mapping algorithms (R/qtl, QTL Cartographer, and WebQTL) to map QTLs in the LXS, and we compared the results. Most mapping studies use only a single algorithm, an approach that may result in failure to identify minor QTLs. We confirmed most of our previously reported QTLs, although one major QTL from earlier work (Lore) failed to replicate, possibly because it represented multiple linked genes separated by recombination in the RI strains. We also report narrowed confidence intervals, based on mapping with a new genetic resource of more than 4000 polymorphic single-nucleotide polymorphism markers. These narrowed confidence intervals will facilitate candidate gene identification and assessment of overlap with human regions specifying risk for alcoholism. Finally, we present an approach for using these RI strains to assess evidence for candidate genes in the narrowed intervals, and we apply this method to a strong candidate, the serotonin transporter.

Initial sensitivity to the intoxicating effects of ethanol is a major risk factor for subsequent development of alcoholism in family history-positive males (Shuckit, 2000). The inbred Long Sleep (ILS) and inbred Short Sleep (ISS) mouse strains are excellent resources for addressing the genetic bases of this behavior, because they were derived by selection for differential sensitivity to a sedative dose of alcohol (McClearn and Kakihana, 1981). The ILS and ISS mice, and their progenitors, have been widely used in alcohol research, and they have been cited in some 400 publications over the past 30 years.

In previous work, we mapped QTLs for sensitivity to an intoxicating dose of ethanol [loss of righting due to ethanol (LORE), Lore, 1–5], using a small panel of 25 RI strains (LSXSS) derived from the noninbred Long Sleep (LS) and Short Sleep (SS) (Markel et al., 1996) mice, and subsequently, a large F₂ intercross between ILS and ISS mice of more than 1000 mice (Markel et al., 1997). The latter cross confirmed four of the putative QTLs from the LSXSS and identified three novel regions. We confirmed and captured four of the major QTLs for this trait, on chromosomes 1, 2, 11, and 15, in reciprocal congenic strains (Bennett et al., 2002b), and we narrowed the interval surrounding the QTL on two chromosomes to less than 12 megabases (Mb) (Bennett et al., 2002a).

There are compelling reasons to use a large RI set such as the recently created LXS set of 75 strains (Williams et al.,...
2004) for genetic mapping (Belknap, 1998; Williams et al., 2004; Chesler et al., 2005): 1) a 4-fold map expansion provides improved map resolution; 2) trait heritabilities are higher in RI than in F2 or backcross animals from equivalent crosses, allowing the mapping of traits with lower narrow-sense heritability \( h^2 \); 3) genotyping is done only once; and 4) phenotypic data from many different laboratories and experiments can be combined to derive genetic correlations and assess gene-environment (GXE) interaction. The study of GXE interactions is uniquely suited to RI sets and is likely to be enormously important in understanding and dissecting complex diseases (Churchill et al., 2004).

Haughney et al. (2005) used the LXS, along with binding assays and molecular evidence from ILS and ISS mice, to identify the norepinephrine transporter (NET) gene on chr 8 as a candidate for a LORE QTL. Here, we present new haplotype data further supporting this candidate.

The serotonin transporter (SERT) is an attractive candidate for ethanol sensitivity in both mice and humans. In a human population, a polymorphism in SERT resulted in a GXE interaction such that stressful events elicited more serious responses in one genotype (Caspi et al., 2003). Mice with one or two null alleles show more anxiety and greater increases in stress hormones following stressful stimuli (Murphy et al., 2001). The gene is located at the peak of the Lore4 QTL on chr 11 (Markel et al., 1997) at 76.7 Mb. The SERT inhibitor fluoxetine has differential effects in ILS and ISS mice on MK-801-induced activity, indicating that 5-hydroxytryptamine (serotonin) affects activity induced by N-methyl-D-aspartate receptor blockade in these mice (Hannahia et al., 2002). An inverse relationship between ethanol consumption and serotonin level has been observed in mice (Kelai et al., 2003), and treatment with selective serotonin reuptake inhibitors has been reported to decrease drinking in alcohol-dependent humans (Lejoyeux, 1996). Here, we tested and ultimately rejected SERT as a candidate for Lore4.

All LORE testing in the LXS panel, reported here, was done in three independent cohorts over a 1-year period. This experimental design was initially developed to spread out specific environmental effects on phenotypic variability to reduce the impact stemming from a single cohort. Nonetheless, environmental effects were large, and some variability in QTLs among cohorts occurred. Here, we report QTLs based on analyses of the individual cohorts and the combined data set. Although the multiple cohort design did not minimize the effect of nonspecific environmental variation, it did allow us to use the second cohort to estimate effect size more accurately (Bennett and Carosone-Link, 2006). Several QTLs that replicated across cohorts, and previous studies, attained high combined significance levels, confirming them as important genomic regions for follow-up work in gene identification.

**Materials and Methods**

**Animals.** The LXS RI panel was generated in the specific pathogen-free (SPF) facility at the Institute for Behavioral Genetics (Boulder, CO) from crosses between the ILS and ISS mice (Bennett et al., 2002b; Williams et al., 2004). The number of consecutive filial matings currently ranges from F27 to F33, with 75 strains still extant. At 25 days of age, mice were weaned, tail clipped, and transferred to male-only or female-only cages, two to five per cage. Mice were maintained on a 12-h light/dark cycle and were given food and water ad libitum (Breeder Diet, 9% fat; Harlan, Indianapolis, IN). All procedures followed guidelines developed by the National Institutes of Health and the Institutional Animal Care and Use Committee.

**Loss of Righting Reflex Due to Ethanol.** All testing was conducted in the SPF facility. Mice were tested for alcohol sensitivity first at 55 to 65 days (trial 1) and again 7 days later (trial 2) by i.p. injection of a 4.1-g/kg dose of ethanol [20% (w/v)] solution in saline. Blood ethanol concentration (BEC) at awakening was determined using a spectrophotometric assay (Smolen and Smolen, 1989). All testing was done between 9:00 AM and 2:00 PM, during the light cycle. If the mouse still had not lost the ability to right itself, after 10 min, the injection was considered faulty, and a retest was done 1 week later. Approximately 10% of all mice injected failed to lose the righting response on one occasion; this is generally attributed to a misplaced or leaky injection and is a typical occurrence for i.p. injections (Markel et al., 1995; Crabbe et al., 2005). Blood ethanol concentration was determined in 66% of these animals 10 min postinjection to corroborate this conclusion; these data are presented here. Duration of LORE was determined using the method of McClearn and Kakihana (1981), modified as reported by Markel et al. (1997). In brief, righting response was lost when the mouse could not right itself three times within 1 min. All mapping was done using the mean LORE duration from trials 1 and 2. ISS and ILS mice were injected as controls for environmental variability in LORE.

**[H]Citalopram Binding to SERT.** Mice were sacrificed by cervical dislocation. Brain regions of interest (prefrontal and remaining cortex, cerebellum, hippocampus, nucleus accumbens, amygdala, ventral midbrain, and caudate) were dissected from ILS and ISS mice \( n = 6–8 \) (strain) and frozen at \(-80^\circ C\). On the day of assay, tissues were homogenized in 30 mM sodium phosphate buffer, pH 7.4, containing 0.32 M sucrose and centrifuged at 20,000g at 4°C for 20 min. The membrane pellets were resuspended in the phosphate-sucrose buffer and incubated in a volume of 0.25 ml with \([H]citalopram\) (PerkinElmer Life and Analytical Sciences, Boston, MA), unlabeled citalopram, and fluoxetine (Sigma-Aldrich, St. Louis, MO) at room temperature for 1 h. Indirect saturation curves were generated in “remaining cortex” and cerebellum using 4.7 nM \([H]citalopram\); concentrations of unlabeled citalopram ranging from 0.03 nM to 1 \(\mu M\), and \(-80 \mu g\) of protein. Nonspecific binding was defined with 10 \(\mu M\) fluoxetine. In the other brain regions, specific binding was measured with a single, 2-fold higher concentration of \([H]citalopram\) (9.5 nM \(\pm 10 \mu M\) fluoxetine) and \(-75 \mu g\) of protein. The assays were terminated by rapid vacuum filtration over GF/B filters (Brandel Inc., Gaithersburg, MD) and three washes with ice-cold sodium phosphate buffer. The retained radioactivity was measured by liquid scintillation spectrometry. Proteins were determined by the method of Bradford (1976) using bovine serum albumin as the standard. The maximal number of binding sites \( B_{MAX} \) and affinity \( K_D \) were determined from the saturation curves with nonlinear fitting (GraphPad Software Inc., San Diego, CA).

**Heritability Determination and Statistical Analyses.** Narrow-sense heritability \( h^2 \) assesses the proportion of the phenotypic variance \( (\text{VP})\), due to additive genetic variance \( (\text{VA})\). \( h^2 \) is easily determined in an RI study as the variance of the strain means, relative to the total variance, and was calculated separately for sex and cohort by adjusting \( r^2 \) from a one-way analysis of variance for varying sample sizes (Belknap et al., 1996). The 95% confidence limits on \( h^2 \) were determined using the Moriguti-Bulmer procedure for approximating confidence limits (Sokal and Rohlf, 2000). Statistical analyses were done using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL).

**QTL Mapping and Haplotype Analysis.** QTL mapping was done in several stages, to identify loci acting individually and QTLs that interacted, either additively or epistatically, to affect each phenotype. Initial analysis was done with MapManager QTXT, version 19 (Manly and Olson, 1999; Manly and Cudmore, 2001; http://www.mapmanager.org/mmtQTXT.html), to produce a genetic map that was then used as the input file for R/qtl. The genetic map was based on genotype determined previously for the LXS strains (Williams et al.,
LORE in the LXS. In total, 952 mice in three cohorts were tested for LORE (Table 1). The third cohort included only 206 mice from 57 strains, providing low power for mapping QTLs; consequently, no individual analyses of this cohort are reported, although we did use these data in pooled analyses. Correlations among all three cohorts, along with heritabilities, are given in Table 1, and they indicate good phenotypic correspondence in all three cohorts. At the time testing began, there were 77 strains extant; three have since been lost. There was no transgression, that is, no strain means for LORE surpass either of the progenitor strains (Fig. 1). This is an optimal situation for genetic mapping of the selected trait (Liu, 1997). LORE in strain 35 ($\mu = 20.5$) was as low as that in ISS ($\mu = 22.5$; $p = 0.84$), and ILS mice had the longest duration of LORE ($\mu = 185$), significantly greater than the next highest strain ($\mu = 149$; one-tailed $p = 0.04$). Heritability of LORE, based on the RI strain means in the combined data set, was 57%. Heritability values by sex and cohort all clustered close to this value (Table 1).

BEC at awakening was determined for 814 mice with successful injections. As reported previously (DeFries et al., 1989; Markel et al., 1997), this measure was significantly negatively correlated with LORE ($r = -0.41$ for the first determination of LORE, $p < 0.001$; $r = -0.43$ for the second determination of LORE, $p < 0.001$). BEC at awakening assesses central nervous system sensitivity to ethanol: ISS recover the righting response at a much higher BEC than ILS strain. Any between-strain difference in ethanol metabolism is primarily due to dose and administration (Smolen et al., 1986), neither of which varied here. Heritability of BEC is much lower than for LORE ($-0.2$ in the present study, due to error variance in the test), making this trait less desirable for mapping. To rule out any confounding effect of BEC in mapping QTLs for LORE, the strain mean was regressed out, and residuals were correlated with LORE. The correlation was highly significant ($r = 0.97$; $p < 10^{-40}$), indicating that no correction for BEC was necessary.

For all strains but ISS, failure to lose the righting response within 10 min was most likely due to a faulty injection. Mean BEC for these mice was 323 mg/100 ml (Fig. 2A), statistically lower ($p < 0.001$) than in mice that did lose the righting response ($\mu = 357$ mg/100 ml), although this latter value is BEC at awakening, which will be somewhat lower than BEC at loss of righting. Consequently, data from animals failing to lose the righting response were not used in determining strain mean for LORE. In ISS, a different picture emerged. In a subset of these mice, low BEC undoubtedly followed a misplaced injection (Fig. 2B, columns on the left; $\mu = 199$

### Table 1

Heritability* by sex and cohort and correlations (p value) for LORE

<table>
<thead>
<tr>
<th></th>
<th>Males, Coh 1</th>
<th>Males, Coh 2</th>
<th>Females, Coh 1</th>
<th>Females, Coh 2</th>
<th>Males, Coh 3</th>
<th>Females, Coh 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males, Coh 1</td>
<td>0.68</td>
<td>0.59*</td>
<td>0.76*</td>
<td>0.70*</td>
<td>0.72*</td>
<td>0.75*</td>
</tr>
<tr>
<td>Males, Coh 2</td>
<td>0.53</td>
<td>0.68*</td>
<td>0.82*</td>
<td>0.77*</td>
<td>0.72*</td>
<td>0.72*</td>
</tr>
<tr>
<td>Females, Coh 1</td>
<td>0.66</td>
<td>0.71*</td>
<td>0.77*</td>
<td>0.81*</td>
<td>0.81*</td>
<td>0.81*</td>
</tr>
<tr>
<td>Females, Coh 3</td>
<td></td>
<td>0.56</td>
<td>0.75*</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (Strains, mice)</td>
<td>(76,182)</td>
<td>(70,193)</td>
<td>(73,194)</td>
<td>(66,177)</td>
<td>(45,99)</td>
<td>(47,107)</td>
</tr>
</tbody>
</table>

*Correlations significant at $p < 10^{-3}$; all others, $p > 0.05$. Heritabilities are given on the diagonal. For combined cohorts, $h^2 = 0.51$ for males, 0.63 for females, and 0.57 for combined sexes.
resulted in a floor effect. To the sedative effect of ethanol in the short sleep mice (Crabbe et al., 2005), indicating that selection for resistance LS (Smolen and Smolen, 1989) and most inbred strains (mg/100 ml). In a second group, BEC was significantly less than BEC at awakening in mice in the righting response. A, less sensitive, non-ISS strains were pooled. Two groups emerged: those consistent with a faulty injection (left), which had significantly lower BEC than those apparently resistant (right) to the sedative effect of the administered dose (4.1 g/kg). Pooling over cohorts identified all QTL regions albeit with slightly lower LODs. The number of strains tested showed a suggestive correlation with LOD score (r = 0.34; p = 0.09). No sex-specific QTLs were identified.

Identifying LORE QTLs. Because within-cohort correlations were larger than between-cohort correlations, cohorts 1 and 2 were initially analyzed separately; subsequently, all data were pooled over cohorts. The QTL region on chr 1 was identified in both cohorts (Table 2). Regions on chr 3, 8, 14, and 18 emerged in one cohort but not the other (Table 2). Pooling over cohorts identified all QTL regions albeit with slightly lower LODs. The number of strains tested showed a suggestive correlation with LOD score (r = 0.34; p = 0.09). No sex-specific QTLs were identified.

All mapping programs gave very similar findings for QTLs (Fig. 3, R/qtl (A) and QTL Cartographer (B); Supplemental Fig. 1, WebQTL; and Supplemental Fig. 2, QTL Cartographer). Figure 3 illustrates discrepancies among the mapping programs. For example, R/qtl (Fig. 3A) identified the chr 3 QTL as significant (p = 0.05), whereas this region is suggestive in QTL Cartographer (Fig. 3B). Likewise, regions on chr 14 and 18 are suggestive in R/qtl (Fig. 3A) but not in QTL Cartographer (Fig. 3B). QTL regions on chr 1, 3, 8, 14, and 18 replicated in one or both previous mapping populations with highly significant combined p values (Markel et al., 1996, 1997). In the LSXSS RI panel, QTLs were identified by single marker t tests, which did not permit estimation of confidence intervals. For these regions, replication is surmised based on inclusion of the point estimate from Markel et al. (1996) in the 1-LOD interval from the LXS. The chr 1 QTL replicated in all three mapping populations, with the highest combined p value, and also confirmed in congenic strains (Bennett et al., 2002a,b). The 1-LOD intervals reported here (for this chr 1 QTL) do not quite overlap with QTLs from the F₂ intercross (Markel et al., 1997). It is worthwhile noting that the F₂ estimate of the peak LOD is at 90 Mb and the 1-LOD interval spans 74 to 93 Mb, suggesting that there was an error in determining this interval. Although confidence intervals are not necessarily normal, a less skewed distribution relative to the peak LOD would indicate a support interval of approximately 74 to 106 Mb, and the Lore region identified in the F₂ would overlap with that in the LXS.

Replication across Mapping Programs. We compared suggestive (p < 0.63) and significant (p < 0.05) QTLs from interval analysis from all three mapping packages (Table 3; full graphics from WebQTL and multiple trait mapping analyses from QTL Cartographer are provided in Supplemental Figs. 1 and 2, respectively). Sexes were analyzed separately, pooling data over cohorts 1 and 2, because the data are archived in this format in WebQTL. The QTLs on chr 1, 3, and 14 replicated in all algorithms, although significance levels differed. There was a surprising range of significance levels. For example, regions on both chr 1 (males) and 3 (females) approached significance in R/qtl and Cartographer but not in WebQTL (Table 3). However,usal of the LODs partially resolves the dilemma; all LODs are quite close but
the reported p values differ. This disparity is likely due to the fact that WebQTL reports only suggestive, significant, or highly significant cut-offs, whereas in the other programs it is possible to obtain a more precise probability. In addition, WebQTL defaults to 2000 permutations for estimating significance cut-offs, whereas in other programs 1000 permutations were done. In R/qtl and Cartographer, there was little difference between the significance cut-offs obtained using 1000 or 2000 permutations. Despite this variation, general trends over packages were very similar, with the largest disparity in WebQTL. For example, the chr 1 QTL in males had lower LOD scores than females in all programs. For chr 3, the direction of the difference was reversed, with males having higher LODs than females. The peak position and support interval for QTLs on chr 1 and 3 were extremely similar among all mapping programs, although the chr 14 region varied considerably. A suggestive region on chr 18 was identified in males only in R/qtl and suggested in both sexes by a significant GXE interaction determined by multiple trait mapping in Cartographer (Supplemental Fig. 2). A suggestive region on chr 19 was identified by both WebQTL and Cartographer. In all analyses of variance, strain contributed significantly (p < 0.001) to LORE. Although two to three different investigators tested LORE in each cohort, this variable did not have a significant effect on LORE for either sex.

### 1-LOD Intervals Are Reduced in the LXS

The 1-LOD support intervals in the RI panel (LXS columns) were reduced relative to those obtained in the F2 map (Table 2), with the exception of the chr 1 region. (And as noted above, the determination of this interval in the F2 is suspect.) These intervals were reduced even further when the denser SNP map, archived on the WebQTL site, was used (Table 4). On average, using the denser SNP map reduced the 1-LOD support interval 4-fold, relative to the microsatellite map. The range in absolute values was quite remarkable: the chr 14 region showed a support interval of 2.9 Mb in males (down from 40.8 Mb using microsatellites), whereas the chr 3 region, 18.3 Mb by microsatellite mapping, was reduced to approximately 9 Mb using the SNP map.

### Composite Interval and GXE Mapping

Controlling for all suggestive and significant background QTLS reported in Table 2 (composite interval mapping in QTL Cartographer) resulted in a large increase in the significance of the QTLS on chr 1 and 3 in both sexes, such that both of these regions showed a highly significant probability of containing a QTL for LORE (p < 0.001 for females and p < 0.01 for males). Although a number of other QTL regions were suggestive by this method, none of them surpassed the significance cut-off determined by 1000 permutations. Including independent sex- and cohort-specific LORE phenotypes in a multiple trait mapping analysis also provides a likelihood ratio statistic for GXE interaction, because the same phenotype was measured in different environments (i.e., cohorts and sexes). Significant GXE interaction was seen on chr 4, 8, 10, 11, and 15, indicating genomic areas that affected LORE in specific environments (Supplemental Fig. 2).

For each cohort, a model was developed including interacting loci that together accounted for a sizable portion of the phenotypic variance (Table 5, top). These loci were identified from a two-way scan of strain means and locus genotypes in R/qtl and selected on the basis of permutation tests for joint LODs. None of these interactions exceeded the permutation cut-off for epistasis, indicating only additive effects among the loci, which were the same in each cohort (Table 5, bottom). For cohort 1, these three loci, on chr 1, 3, and 8, acting additively, accounted for more than 40% of the phenotypic variance, whereas for cohort 2, this value was 30%.

### Table 2

<table>
<thead>
<tr>
<th>Chr, Mb Position</th>
<th>LXS Cohort 1</th>
<th>LXS Cohort 2</th>
<th>Pooled Cohorts*</th>
<th>F2†</th>
<th>LSXSS RI‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOD (p Value)</td>
<td>[1-LOD Interval Mb]</td>
<td>LOD (p Value)</td>
<td>[1-LOD Interval Mb]</td>
<td>LOD (p Value)</td>
</tr>
<tr>
<td>1, 134.22*</td>
<td>2.88 (0.1)</td>
<td>[103.4–154.1]</td>
<td>2.41 (0.15)</td>
<td>[103.4–173.1]</td>
<td>2.8 (0.1)</td>
</tr>
<tr>
<td>3, 143.60*</td>
<td>3.28 (0.04)</td>
<td>[133.4–151.7]</td>
<td>1.41 (&gt;0.63)</td>
<td></td>
<td>3.1 (0.05)</td>
</tr>
<tr>
<td>8, 118.34*</td>
<td>(NS)</td>
<td></td>
<td>1.87 (0.63)</td>
<td>[102.0–128.3]</td>
<td>(NS)</td>
</tr>
<tr>
<td>14, 90.66</td>
<td>2.77 (0.15)</td>
<td>[71.0–97.7]</td>
<td>(NS)</td>
<td></td>
<td>2.3 (0.3)</td>
</tr>
<tr>
<td>18, 89.33*</td>
<td>2.78 (0.15)</td>
<td>[77.0–89.2]</td>
<td>(NS)</td>
<td></td>
<td>2.3 (0.3)</td>
</tr>
</tbody>
</table>

* Data from cohorts 1 and 2 were pooled before analysis.
† Markel et al. (1997).
‡ Markel et al. (1996).
§ Effect size adjusted by analysis in two cohorts, one cohort for location and the second cohort for effect size (for details, see Bennett and Carosone-Link, 2005).
© Confirmed (chr 1, 18) or identified (chr 8) in F2 population.
| Strains (mice) | 76 (376) | 72 (370) | 77 (952) | (1073) | 27 (1114) |

Fig. 3. Interval mapping results for LORE from R/qtl (A) and QTL Cartographer (B), sexes and cohorts combined.
The following experiments were done to test the hypothesis that *Slc6a4* was a candidate gene underlying some of the difference in LORE between ILS and ISS. Specific binding of \(^{3}H\)citalopram, a highly selective SERT inhibitor (Hyttel, 1994), to crude membranes prepared from brain regions of interest, was used to determine whether there were any regional differences in the number of SERTs between the ILS and ISS mice. Full saturation curves in cerebral cortex (minus prefrontal cortex) and cerebellum showed a single binding site with high affinity (\(K_D\) values of ~3 nM) and \(B_{MAX}\) values of ~500 fmol/mg protein, but no strain differences. Likewise, specific binding of 9.5 nM \(^{3}H\)citalopram to membranes prepared from other brain regions showed the regional differences in levels of SERTs (ventral midbrain, ~500 fmol/mg protein; nucleus accumbens, ~480 fmol/mg protein; amygdala, ~480 fmol/mg protein; dorsal striatum, ~360 fmol/mg protein; hippocampus, ~320 fmol/mg protein; and prefrontal cortex, ~230 fmol/mg protein) but no differences between the ILS and ISS mice.

Haplotype analysis suggested that ILS and ISS are not polymorphic through *Slc6a4* (boldface in Table 6), although ILS and ISS were not genotyped in the gene itself, they and all other strains but B6 share the same haplotype over the 107 SNPs in the SERT gene. The B6 is different from the other two strains. The divergence of B6 both upstream (10 SNPs) and in the gene suggests that ILS and ISS do not possess this haplotype but instead share a nonpolymorphic haplotype with the other strains, thus ruling out *Slc6a4* as a candidate.

Haplotype analysis of 78 SNPs in the NET gene (*Slc6a2*) revealed substantial polymorphism (Fig. 4). ILS, D2, and A share one haplotype. B6 has a different haplotype upstream, and in the proximal region of the gene. Through the distal portion of the gene (exon 4 and 11), all strains but ISS share a common haplotype. ISS is B6-like in the proximal region of the gene, but it has a unique SNP pattern in exons 4 and 11.

**Discussion**

As expected (Belknap, 1998), heritability for LORE in the LXS was substantially higher (Table 1) than our previous estimate of ~0.4 (Markel et al., 1997). Increased \(h^2\) facilitates...
genetic mapping, but variability in behavioral phenotypes can cause problems in QTL analysis. A cohort design may amplify environmental effects not common to both cohorts, resulting in identification of different QTLs due to specific GXE interactions. This approach may be necessary because of limitations on space or resources, and it is preferable to testing all individuals of a given strain in one block. This effect is well known in the plant literature (Beavis, 1994) but less so in mammalian QTL work where independent mapping populations are less common. We suggest that the slight differences in mapping results shown in Table 2, identified in cohorts 1, 2, and 3, are due to this effect (Bennett and Carosone-Link, 2006). Despite a few between-cohort differences, in general, the QTLs replicated remarkably well considering that each accounted for less than 10% of the phenotypic variance. This replication indicates that the QTLs are real; however, p values (or LODs) are not overwhelming as the effect size of each is small.

Mapping using the combined data resulted in replication of virtually all QTLs from the individual cohort analyses, with slightly lower LOD scores (Table 2) and effect sizes (Bennett and Carosone-Link, 2006). Environmental effects specific to a single cohort were averaged over the unaffected mice in the other cohorts, decreasing the significance of cohort-specific QTLs (Table 2). Thus, this approach may provide more robust identification of QTLs operating across most conditions. A preferred approach involves resampling the pooled data, randomly assigning all values to one of two cohorts: the first cohort for estimating QTL location, and the second cohort for estimating effect size (Melchinger et al., 1998). All but one of the QTLs identified in the original data set replicated in the resampling design with higher LODs and larger effect sizes (Bennett and Carosone-Link, 2006).

Although QTL regions on chr 1 and 3 were significant for one sex but only suggestive for the other sex, there were no completely sex-specific QTLs. This result is not surprising, given the consistent difference between LORE in males and females, reported in a previous study (Markel et al., 1995). This differential sensitivity is due, in part, to differences in body fat and aqueous compartments between the sexes, which alter ethanol distribution (Goldstein, 1983), and slightly higher elimination rates in females (Owens et al., 2002).
relating to nonreplicability, which are important to recognize for follow-up confirmation studies. Although the chr 14 region was identified in cohorts 1 and 3 (Table 2), and in all three mapping packages (in at least one sex), the support intervals do not overlap. This ambiguity makes it difficult to pursue this QTL, despite its overlap with a human QTL for ethanol sensitivity (Schuckit et al., 2005). The chr 18 and 19 regions illustrate a different problem. The chr 18 region was identified by R/qtl in cohort 1, and in previous mapping studies (Table 2) as well as by Cartographer. However, WebQTL did not find this region even suggestive, highlighting the importance of using multiple approaches for mapping. R/qtl did not pick up the suggestive region on chr 19, identified in females by WebQTL and Cartographer.

All but one (on chr 14) of the LORE QTLs reported here replicated regions identified in previous mapping populations (Table 2). This replication, assessed by overlapping 1-LOD support intervals, allowed us to combine p values for each QTL. For the regions on chr 1 and 3, these p values were highly significant (p < 0.001). Composite interval mapping also identified these two QTLs as significant, as did the additive model shown in Table 5. In both cohorts analyzed separately, the same three loci, on chr 1, 3, and 8, explained much of the phenotypic variance. One of these intervals, on chr 1, also contains one of 15 genes with significant differences in expression between ILS and ISS in cerebellum (Maclaren et al., 2006) and ventral tegmentum (M. Miles, personal communication). X-ray repair complementing defective repair in Chinese hamster cells (Xrcc5) has a 2.5-fold higher expression in ISS strain and a human ortholog in a genomic region linked to ethanol sensitivity in humans. A study using data from more than 700 individuals collected by the Collaborative Study on the Genetics of Alcoholism (http://www.niaaa.nih.gov/ResearchInformation/ExtramuralResearch/SharedResources/projcoa.htm) identified several markers on chromosome 2 linked to sensitivity in this population. Xrcc5, the human ortholog of Xrcc5, maps within 2 Mb of the marker with the highest linkage score (Schuckit et al., 2001).

Both F2 mapping (Markel et al., 1997) and interval-specific congenic recombinant strain (ISCR) confirmation (Bennett et al., 2002a) identified a slightly more proximal region on chr 1 than did the LXS. Of six ISCR, four carried a proximal region of ILS on an ISS background, whereas two carried the more telomeric ILS region, identified in the LXS. All ISCR showed the same pattern, of increasing LORE; however, larger sample size in the four proximal strains provided stronger support for this region. The LXS results reported here suggest a second, linked QTL for LORE. The abundance of support for the chr 1 and 3 regions: replication in all cohorts, and all mapping programs, as well as significant LOD scores by composite interval mapping, multiple trait mapping for chr 1, and differential expression for Xrcc5, favor these two as worthwhile of follow-up study.

It is clearly desirable to use the dense SNP map (archived on the Wellcome Trust site at http://zeon.well.ox.ac.uk/rmottbin/strains.cgi). LOD scores were somewhat higher (Table 3), but the main advantage to using the SNP genotypes is the large (on average, 72%) reduction in the confidence interval surrounding the QTL. This reduction will facilitate congenic (Bennett et al., 2002b) and ISCR strain (Bennett et al., 2002a) construction.

Two of the previously confirmed QTLs for LORE, on chr 11 and 15 (Markel et al., 1997; Bennett et al., 2002b), were identified in the LXS only in a multiple trait model, which gains increased power from correlations among individual traits. The significant GXE interactions for these regions indicate their specificity to as yet unknown environmental effects. An RI panel of 75 strains can reliably detect QTLs accounting for 10% (Valdar et al., 2003) to 20% of VG (Belknap, 1998); thus, it is not surprising that not all QTLs replicated in all mapping populations.

The QTL on chr 8 was initially identified in a large F2 intercross (Lore3; Markel et al., 1997) and replicated in two of the three LXS cohorts (Table 2) by all three mapping packages. The 1.5 LOD support interval included the NET gene (Slt6a2), at 92.2 Mb. Knockout mice lacking dopamine β-hydroxylase cannot synthesize norepinephrine (NE), and they are hypersensitive to the sedative effects of ethanol (Weinshenker et al., 2000), making it a reasonable candidate for Lore3. This effect is blocked by acute replacement of central NE. ILS and ISS strains differ in a number of NET characteristics, including Slc6a2 haplotypes, [*H]+NE uptake, NET binding, and mRNA levels, which are consistently 30 to 50% lower in ILS strain (Haughey et al., 2005). NET genotype significantly (p = 0.04) affected LORE in the LXS RI strains, explaining 5.6% of the phenotypic variance, with strains ILS in the NET region sleeping an average of 14 min longer than strains with an ISS genotype. This relatively small effect is likely the reason that Lore3 was the only QTL region from the F2, mapping that failed to confirm in reciprocal congenic strains (Bennett et al., 2002b).

The SERT gene is located at the peak of the Lore4 QTL, and numerous studies have implicated serotonin in ethanol-related behaviors. A suggestive difference in LORE in the LXS further supported SERT as a candidate. The initial SNP analysis suggested that ILS and ISS strains were polymorphic through the gene region, but more recent data argue against this conclusion. The denser haplotype (Table 6) suggests that there are no ILS/ISS polymorphisms in the gene; however, because as no SNPs in the gene were typed in these strains, this conclusion is inferred based on flanking regions. This and the lack of binding differences rule against SERT as a candidate. NET remains as a strong candidate, based on the numerous polymorphisms between ILS and ISS in the gene, particularly in the unique haplotype seen in exons 4 and 11.

LORE constitutes a mouse model with strong face validity to a major risk factor for alcoholism in humans (Schuckit, 2000). Our mapping results in the LXS RI panel provide additional support for the heritable nature of this trait in mice and replicated many previously identified QTLs. Several of these QLTs have now emerged as candidates for intense follow-up to pursue the underlying gene(s) based on their replicability, significance, and interval reduction.

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