RESEARCH

Confirmation of Quantitative Trait Loci for Ethanol Sensitivity in Long-Sleep and Short-Sleep Mice

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Initial insensitivity to alcohol is a strong predictor of human alcoholism, a widespread and heritable health problem. The Long Sleep and Short Sleep lines of mice were developed by genetic selection for high or low alcohol sensitivity. We have identified seven quantitative trait loci (QTLs) specifying differences in alcohol sensitivity using intercross progeny from these selected strains. These QTLs (*Lore1–Lore7*) together account for ~60% of the total genetic variance for this trait. This represents the first report of linkages for genes influencing alcohol action in any mammalian system using stringent, genome-wide mapping criteria.

At least 10 million Americans are reported to have drinking problems. Alcoholism is a complex trait with a significant genetic component and heterogeneous architecture in family studies, suggesting that different genes may contribute to the segregation of alcoholism in different families (Devor and Cloninger 1989). Alcohol sensitivity is an important predictor of alcoholism: Sons of alcoholic fathers are at an elevated risk for alcoholism and are less sensitive to the effects of ethanol than are sons of nonalcoholic fathers (Schuckit 1985, 1994; Newlin and Thomson 1990; Pollock 1992). Moreover, Schuckit (1994) has shown that insensitivity to ethanol is a highly significant predictor of future alcohol abuse in young men, independent of familial alcoholism. It is possible that initial insensitivity to ethanol is a predisposing factor for alcoholism. Thus, a first step toward the understanding of alcoholism may involve the discovery of those genes that influence initial ethanol sensitivity.

The considerable experimental difficulties in studying the genetics of human alcoholism are circumvented by several rodent models developed by genetic selection for various aspects of alcohol action (Crabbe et al. 1994a). Long-Sleep (LS) and Short-Sleep (SS) mice are selected lines that differ 18-fold in ethanol sensitivity, measured as the duration of loss of righting reflex (LORR) after a hypnotic dose of ethanol (McClearn and Kakihana 1981). This differential response to ethanol is mediated primarily by the central nervous system (Heston et al. 1974; Erwin et al. 1976; Howerton et al. 1983) and very little by the rate of ethanol metabolism (Smolen et al. 1986; Smolen and Smolen 1989). LORR is ~40% heritable and is specified by approximately seven quantitative trait loci, or QTLs (Dudek and Abbott 1984; DeFries et al. 1989; Markel et al. 1995a).

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RESULTS

Genetic Strains and Crosses

We have focused on identifying QTLs that influence differential duration of LORR between LS and SS mice (Johnson et al. 1992; Markel et al. 1996b). Previously, we identified 12 provisional QTLs for LORR in the LS × SS recombinant inbred strains (RIs) (Markel et al. 1996). Only one of these (chromosome 2, 80 cM) exceeded confirmational threshold ($P > 0.0002$; 11). In this report we use a large sample of intercross progeny (F_2) derived from reciprocal crosses of the inbred LS (ILS) and the inbred SS (ISS) strains to confirm or disprove provisional QTLs from the RIs (Belknap 1992; Johnson et al. 1992; Neumann 1992) as well as identify new QTLs in a genome-wide screen.

This sample of ILS × ISS F_2 mice ($n = 1072$) was characterized using a repeated measures design

7:92–99 ©1997 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/97 $5.00

wherein LORR was measured on each individual mouse twice separated by ~1 week (Markel et al. 1995a,b); mean LORR did not differ significantly between trials. The use of averaged individual scores resulted in a 39% reduction in environmental variance in the measure of LORR (Markel et al. 1995a), which enhances the resolution of genetic variance for QTL mapping (Falconer 1989).

Genetic Mapping

Ninety-two of the longest and 92 of the shortest sleeping F_2 mice, based on averaged scores, were chosen for genotyping. The 12 QTLs provisionally identified in LS × SS RI strains (Markel et al. 1996) were tested by assessing simple sequence length polymorphism (SSLP; Dietrich et al. 1992) marker genotypes in these regions of the genome (Table 1) using MapMaker/QTL (Lander et al. 1987). Only two provisional QTLs identified previously in the LS × SS RIs were confirmed. These two QTLs had lod scores in F_2 mice of 5.4 and 5.8 near the position of the maxima identified in the RI screen; these values exceed those required for whole genome scans (Belknap 1992; Neumann 1992; Lander and Schork 1994; Lander and Kruglyak 1995). The 1-lod-support interval for each of these QTLs included the locus identified in the RI analysis. These confirmed QTLs are on chromosomes 1 and 2 and show maximum lod scores of 5.4 and 6.6 at 54 and 85 cM, respectively (Table 2; Fig. 1). One other QTL, on chromosome 18, was confirmed but at a lower level of certainty (lod = 1.8, corresponding to a value of 5% error over all tests) (Belknap 1992; Neumann 1992).

Genome Scan

We then conducted a whole genome scan at an average spacing of 20 cM using SSLP markers in the longest and shortest sleeping F_2 mice. Because we used selected strains for the progenitors, the test of significance is one-sided and the likelihood for each QTL found exceeded the recommended threshold that corresponds to a genome-wide significance level of 5% (lod ⩾ 3.4; Lander and Schork 1994; Lander and Kruglyak 1995). Three new QTLs were detected with lod scores higher than 3.4 (Table 2). Together with the three QTLs mentioned above and one weak QTL identified previously on chromosome 7 (Markel and Corley 1994), seven QTLs have been identified. These have been named *Lore1–Lore7* (Table 2) for loss of righting induced by ethanol. Assuming a strictly additive model for QTL effect, these seven loci account for 59% of the genetic variance in LORR. The total effect explained by these seven QTLs is >130 min of the 170-min difference in LORR between ILS and ISS parental strains.

Blood Ethanol Concentration

We also attempted to confirm the provisional QTLs

Table 1. Tests of Provisional QTLs for LORR in ILS × ISS Intercross

RI (LS × SS)				F_2 (ILS × ISS) (local max. lod)			
Chr.	cM	*t*	*P*	lod[a]	max.[b]	cM	(±1 lod)
1	45	3.14	0.002	5.3	5.4	54	(43–59)
1	99	1.98	0.03	1.0	—	—	—
2	80	4.04	0.0002	5.9	6.6	85	(78–95)
3	74	2.01	0.03	0.4	1.0	31	(cen.–70)
4	56	1.92	0.03	1.2	1.5	72	(39–tel.)
4	74	2.00	0.03	1.5	1.5	72	(39–tel.)
5	72	1.89	0.04	1.0	—	—	—
6	46	2.33	0.02	1.4	1.5	48	(7–70)
12	43	2.18	0.02	1.6	3.1	17	(10–26)
13	25	2.23	0.02	1.5	2.3	10	(cen.–13)
16	41	−2.26	0.02	0.6	1.4	57	(40–tel.)
18	16	2.06	0.03	1.2	1.8	41	(24–tel.)

[a]lod score from F_2 analysis at site of maximum from RI analysis.
[b]lod score at site of maximum in the local genetic region from F_2 analysis (n = 184).

Table 2. QTLs for Ethanol-Induced LORR (from Genome Scan)

QTL	Chr.	cM	Max. lod	1-lod interval[a]	Effect (min)	Percent V_G[b]	No. of mice	Method[c] RI	Method[c] F_2
Lore1	1	54	5.4	43–59	20.9	10.2	186	*	***
Lore2	2	85	6.6	78–95	25.2	14.2	186	*	***
Lore3	8	59	3.4	44–71	19.0	7.7	186		**
Lore4	11	49	6.5	44–56	25.0	12.2	186		***
Lore5	15	46	4.0	32–55	19.0	8.4	186		**
Lore6	18	41	1.8	24–tel.	12.6	3.5	186	*	*
Lore7	7	50	1.7	25–61	10.8	2.7[d]	1063	*[d]	*[e]
Total					133.6	59.1			

[a]Peak lod ± 1 lod interval. Map positions from chromosome committee reports.
[b](V_G) Genetic variance.
[c](*) Suggestive; (**) significant; (***) highly significant (Lander and Kruglyak 1995).
[d]Defries et al. (1989).
[e]Markel and Corley (1994).

for blood ethanol concentration at awakening (BEC) found in LS × SS QTL mapping (Markel et al. 1996a). BEC, which is independent of ethanol dose for each strain, measures CNS sensitivity to ethanol and serves to rule out pharmacokinetic (metabolic) aspects of alcohol action: SS mice wake with higher BEC than LS mice (Smolen et al. 1986). Although LS and SS mice may differ in their ethanol metabolism, this difference is primarily attributable to dose and route of administration (Smolen et al. 1986), both of which were constant in this study. Of the 10 tested, only 1 provisional QTL reached suggestive levels of confirmation in F_2 (data not shown). In part, this is because only ~40% of the mice were also tested for BEC but is consistent with the findings for confirmation of LORR.

A whole genome search for QTLs for BEC revealed six provisional QTLs in the ILS × ISS F_2 mice, with lod scores between 2.0 and 3.0. These provisional QTLs are located on chromosomes 2, 8, 9, 11, 14, and 15. One of these QTLs (chromosome 2, 60 cM) was detected previously in the study of the LS × SS RIs (Markel et al. 1996b). These provisional QTLs together explain a range of 184 mg% for differential brain levels of ethanol at awakening and ~50% of the genetic variance. These provisional QTLs for BEC must be confirmed in independent samples, which are currently under way.

Reliability of the Measure for Alcohol Sensitivity

We also addressed whether the strategy of duplicate assessments of LORR on each F_2 mouse led to a noticeable increase in lod scores (Fig. 1). The lod scores of both $LORR_1$ and $LORR_2$ are similar for most loci and not very different from the lod derived from mapping the average. *Lore2* is an exception in that a significantly higher lod score was obtained in mapping $LORR_1$ than in mapping $LORR_2$. This locus may determine tolerance such that the effect of alcohol would not be observed as readily at the second time point because the expression of this gene has been modified by prior exposure.

Epistasis

Applying segregation analysis, a small proportion of genetic variance for LORR was attributed to an epistatic effect among these ILS × ISS F_2 mice (Markel et al. 1995a). Using two-way analyses of variance, nonlinear interactions between each confirmed LORE QTL and each unlinked marker (including other QTLs) were examined. After adjusting probability values for multiple test using a Bonferroni correction (Markel et al. 1996b), no significant epistatic effects were detected between LORR QTLs.

DISCUSSION

This report represents the first general linkage analysis of alcohol action in any mammalian system using stringent, genome-wide mapping criteria. We have determined approximate map positions for

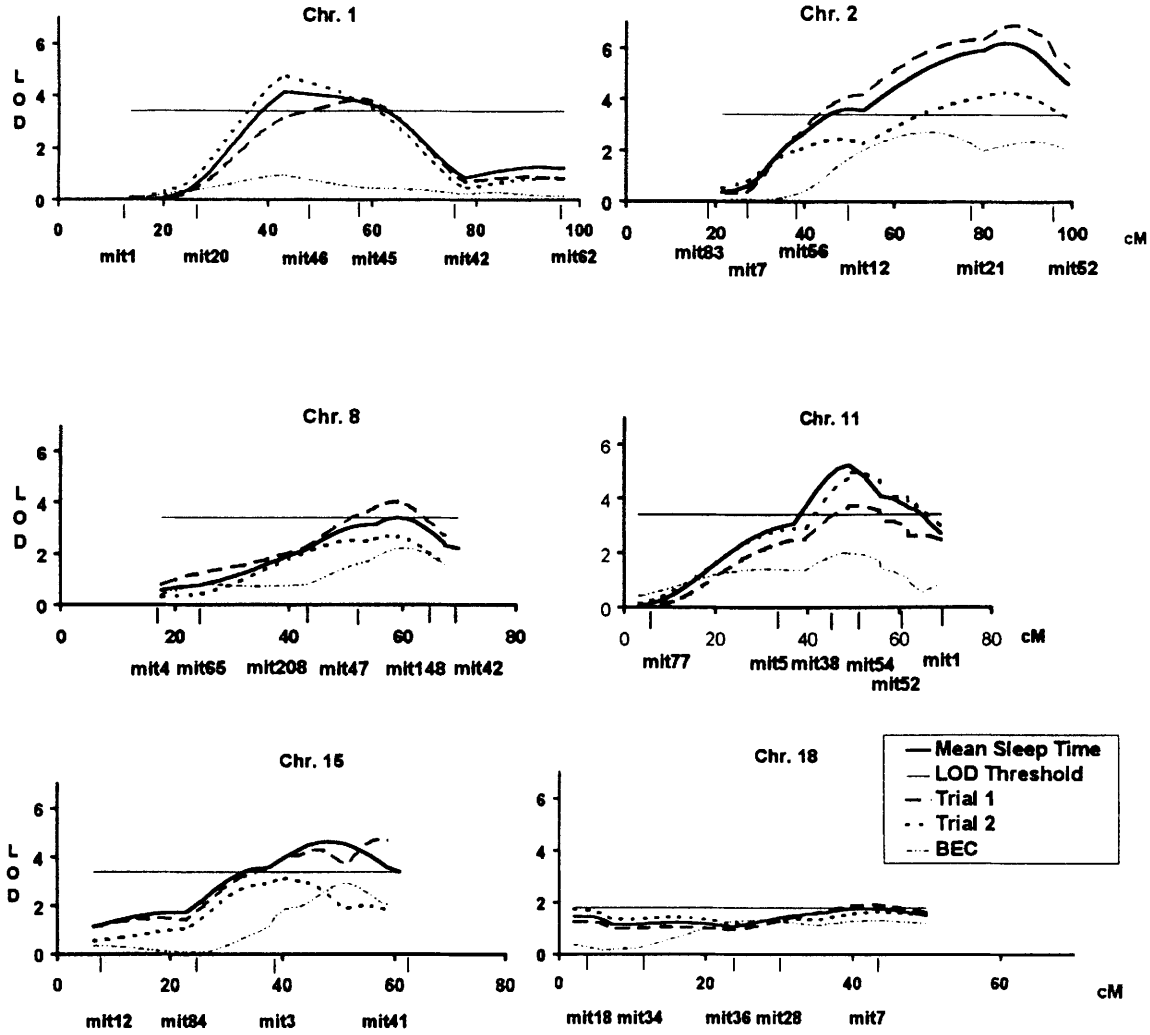

Figure 1 Likelihood maps for QTLs on chromosomes 1, 2, 8, 11, 15, and 18. The lod score is shown on the vertical axis; lod of 3.4 (shown as a horizontal line) is the cutoff for statistical significance in all but chromosome 18, which was confirmed at a lod of 1.8. Genetic distance along the chromosome is plotted on the horizontal axis; markers used for the assessment are also indicated. The thick line is for average sleep time. The two dotted lines show lod scores for sleep time 1 and sleep time 2. A map of BEC is also shown.

seven QTLs that influence individual differences for hypnotic sensitivity to ethanol. The identification of these QTLs for ethanol sensitivity is a valuable step toward identifying genes involved in the etiology of alcoholism and alcohol disorders.

Although several groups have used various RI series for the provisional mapping of QTLs influencing several ethanol related responses (McClearn et al. 1992; Belknap et al. 1993; Crabbe et al. 1994b; Rodriguez et al. 1995; Markel et al. 1996a), our F_2 results strongly encourage confirmation or disproof of provisional QTLs from RI-mapping experiments using large F_2 stocks.

Our findings suggest that initial characterization in RIs can identify major QTLs, such as those on chromosomes 1 and 2, with relative certainty. Nevertheless, three QTLs (*Lore3*, *Lore4*, and *Lore5*) were not detected in RI mapping even though these loci each explain 7%–12% of the variance. This is consistent with earlier power calculations (Belknap 1992; Neumann 1992; Markel et al. 1996a), suggesting that QTLs explaining <25% of the variance in the RI panels of this size would not be detected with any degree of certainty. Equally important is the fact that of 12 other provisional QTLs identified with a probability value between 0.05 and 0.01 (Markel et al. 1996a), only 1 was confirmed in the F_2 replication. The use of RIs alone is likely to produce

mostly false-positive associations unless very stringent statistical criteria are applied.

LORR and BEC are each a measure of ethanol sensitivity. In the LS × SS RI the correlation between LORR and BEC is −0.79 (Smolen and Smolen 1989); in our F_2 it is −0.50. We asked whether the *Lore* QTLs all show the expected negative relationship with BEC (Table 3; Fig. 1). *Lore1* is the only exception to the general finding of significant negative relationships between LORR and BEC. One explanation could be that the *Acrd* and *Acrg* genes, which are in the candidate region for *Lore1* and code for the acetylcholine receptor subunits delta and gamma, respectively, are not expressed in the CNS but in the peripheral nervous system. Instead, *Lore1* may mediate the righting response, in part via peripheral motor control; such nicotinic receptors have been shown to be sensitive to alcohol (Gage et al. 1975; Aiken et al. 1991; Murrell et al. 1991).

Several candidate genes mapped previously can now be explored for possible direct involvement in the etiology of alcohol sensitivity. A number of candidate genes lie within the 1-lod support interval for the *Lore* QTLs (Fig. 2). The gene for the high-affinity neurotensin (NT) receptor (*Ntsr*) is located at ~80 cM on chromosome 2 very near the maximum lod for *Lore2*, and this locus also shows consistent correlation ($P < 0.05$) with the observed NT receptor density in the frontal cortex and striatum in the LS × SS RI panel (V.G. Erwin, P.D. Markel, T.E. Johnson, and B.C. Jones, in prep.). Four of the QTLs reported here (*Lore1*, *Lore2*, *Lore4*, and *Lore5*) fall in virtually the same location as QTLs from the LS × SS RI panel for NT receptor density (V.G. Erwin, P.D. Markel, T.E. Johnson, and B.C. Jones, in prep.). NT treatment enhances hypnotic sensitivity to ethanol in a genotype-dependent manner in the LS and SS lines, and both NT levels and NT receptor density vary widely among the LS × SS RI strains. Densities of the high-affinity NT receptors in the entorhinal cortex, the frontal cortex, and the striatum are positively correlated with ethanol-induced sleep time (Erwin et al. 1993).

The α-adrenergic β2 receptors, via G proteins, activate adenylate cyclase (AC), which has been studied intensively as a biochemical marker for vulnerability to alcoholism (Saito et al. 1987). Ethanol exposure can reduce AC activity as much as 35% in LS mice while effecting no change in SS mice (Wand and Levine 1991). Two candidate genes in this pathway are located at or near *Lore2*; these are *Adra2b*, the α2 adrenoreceptor subtype 2b at 71 cM, and *Gpcr8*, the G-protein-coupled receptor 8, at 73 cM.

Prodynorphin (Pdyn) and inducible nitric oxide synthase (Nos2) have been implicated in alcohol action; these are candidate genes near *Lore2* and *Lore3*, respectively. Other candidate genes of general pharmacological interest are suggested by the mapping data. For example, a serotonin transporter gene (Htt) was found near *Lore4* on chromosome 11. *Cchl1a3* (calcium channel L, type 1A3 subunit) is within the support region for *Lore1*. *Cyp11b-1,-2* (steroid-11-β-hydroxylase) is a candidate for *Lore5* on chromosome 15.

METHODS

Propagation and Characterization of Mice

All animals were developed and obtained from the Institute for Behavioral Genetics, (Boulder, CO). All experiments were

Table 3. Summary of Findings for *Lore* Genes

		LORR		BEC		Individual LORR[a] (trial 1)		(trial 2)	
QTL	Chr.	max. (lod)	effect (min)	max. (lod)	effect (mg%)	(lod)	effect (min)	(lod)	effect (min)
Lore1	1	5.4	22	0.8	N.S.[b]	3.9	20	4.8	21
Lore2	2	6.6	25	2.0	−27	6.9	24	4.3	22
Lore3	8	3.4	19	2.2	−39	4.0	20	2.7	16
Lore4	11	6.5	25	2.0	−37	3.7	23	5.0	24
Lore5	15	4.0	19	3.0	−19	4.3	20	3.2	16
Lore6	18	1.8	12	1.3	N.S.[b]	1.9	11	1.6	13
Lore7	7	1.7	11	2.7	N.S.[b]	2.0	10	1.4	11

[a]Peak lod for first and second measurements of LORR (see text).
[b](N.S.) Not significant.

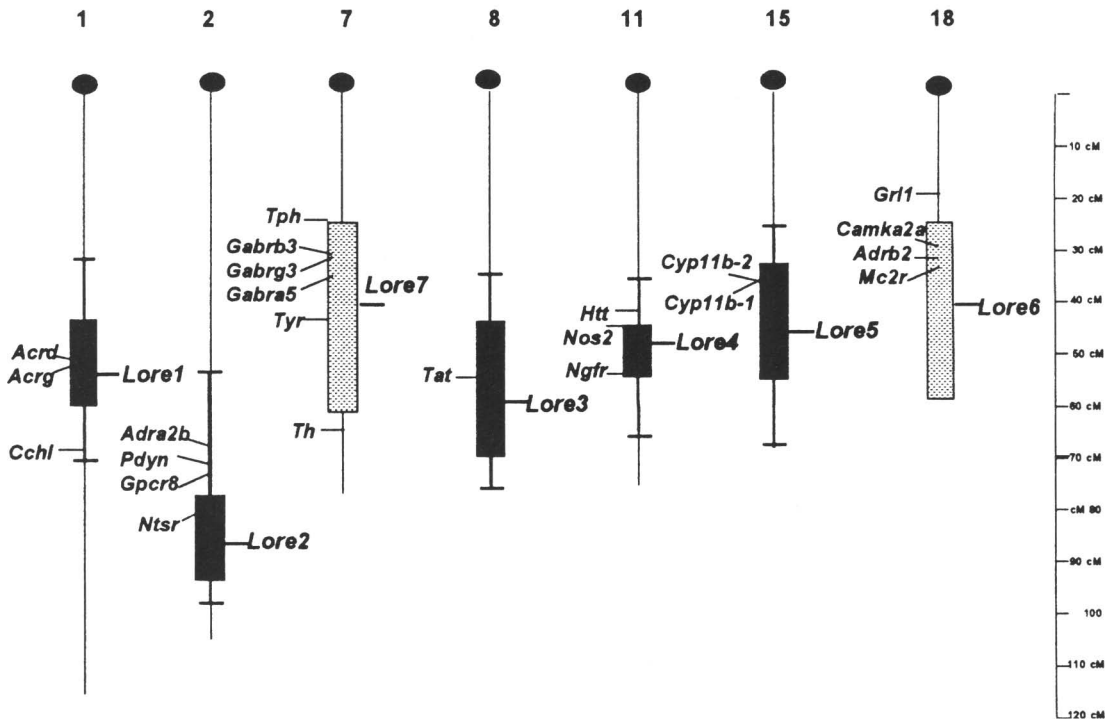

Figure 2 *Lore1–Lore6*. The thick bar shows a 1-lod support interval; thin lines show the 2-lod support interval. Some candidate genes are also shown: (*Acrd*) Acetyl choline receptor subunit δ; (*Acrg*) acetyl choline receptor subunit γ; (*Cchl*) calcium channel L, type 1A3 subunit; (*Adra2b*) a2 adrenoreceptor subtype 2 b; (*Pdyn*) prodynorphin; (*Gpcr8*) G-protein-coupled receptor; (*Ntsr*) high-affinity neurotensin receptor; (*Tph*) tryptophan hydroxylase; (*Gabrb3*) GABA β3; (*Gabrg3*) GABA γ3; (*Gabra5*) GABA α5; (*Tyr*) tyrosinase (*C*, or albino) (*Th*) tyrosine hydoxylase; (*Tat*) tyrosine aminotransferase; (*Htt*) serotonin 5-hydroxytryptamine transporter; (*Nos2*) inducible nitric oxide synthase; (*Ngfr*) nerve growth factor receptor; (*Cyp11b-1*) steroid 11-b-hydroxylase; (*Cyp11b-2*) steroid 11-b-hydroxylase; (*Grl1*) glucocorticoid receptor-1; (*Camk2a*) calmodulin kinase 2a; (*Adrb2*) adrenergic receptor b2; (*Mc2r*) melanocortin 2 receptor.

reviewed and approved by the proper institutional committees.

ILS and ISS mice were developed by 20 generations of sib matings of LS or SS, respectively. At generation 30, ILS females were crossed with ISS males (L/S), and the reciprocal crosses (S/L) were performed to produce the F_1 generation. F_2 mice were produced by four crosses: L/S × L/S, L/S × S/L, S/L × L/S, and S/L × S/L. Mice were maintained on a 12-hr light/dark cycle and were given food and water ad libitum (Wayne diet by Teklad). Mice were tested at 55–65 days of age (trial 1) and again 7–10 days later (trial 2). A 4.1 grams/kg dose of ethanol [20% (wt/vol) solution in saline] was injected intraperitoneally between 9:00 a.m. and 1:00 p.m., 2–6 hr after the start of the light cycle. Mice were injected in the same order on both trials. Duration of LORR was determined by placing mice on their backs in a Plexiglas trough after injection. The start of LORR was considered as the minute when a mouse, after being placed on its back, was no longer able to right itself at least three times within 1 min, and the duration of LORR was the time until spontaneous righting occurred at least three times within 1 min. An LORR score of zero was assigned if no LORR was seen within 15 min after injection. Mice were excluded for leaky or subcutaneous injections. Mice were returned to cages after testing and were subsequently sacrificed for molecular analyses.

DNA Marker Analysis

Some methods were reported previously (Markel and Johnson 1994). Primer pairs that amplify SSLPs were obtained commercially from Research Genetics (Huntsville, AL). The forward primer for each marker pair was labeled at the 5′ end with [γ-^{33}P]ATP. A typical labeling cocktail for 40 reactions was 4.48 mM in the forward primer and contained 5.0 units of T4 polynucleotide kinase (New England Biolabs), and 1.7 ml of [γ-^{33}P]ATP (sp. ac. 2000 Ci/mM; Dupont/NEN) in 10 ml of kinase buffer. Reactions were incubated at 37°C for 30 min and stopped by incubation at 65°C for 10 min. Reaction cocktails (all ingredients minus template) were prepared manually and contained the components from the labeling reactions—0.25 units of *Taq* polymerase (Perkin Elmer Cetus)—and were 112 nM for each primer, 200 mM for each nucleotide, 2.0 mM for $MgCl_2$ (Promega), 10 mM for Tris-HCl at pH 8.4, and 50 mM for KCl. Aliquots (5 μl) of the reaction cocktail were transferred using the P20 pipetting tool of the Biomek 1000 Work-

station (Beckman) into each reaction tube (Perkin Elmer). Aliquots of 5 µl of target DNA at 4 ng/ml were added with the P20 pipetting tool giving 20 ng of genomic DNA in a final volume of 10 ml. PCRs were initiated by 3 min at 94°C, followed by incubation for 30 cycles of 30 sec at 94°C, 20–30 sec at 55°C, and 40–60 sec at 72°C on a Perkin Elmer Cetus 9600 Thermocycler. PCR products were diluted with the addition of 7 µl of a stop-dye solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol in water). This solution was incubated in a 65°C heating block for 2–5 min. Samples were electrophoresed on 5% polyacrylamide denaturing gels (Sequagel; National Diagnostics) for 1.5–2 hr at 50 W. Gels were fixed to filter paper, overlayed with Saran Wrap (Dow Chemical), and dried on a Bio-Rad gel dryer (model 583) for 2 hr at 80°C. Dried gels were exposed directly to film (XAR 5, Kodak) overnight at room temperature. Autoradiographs were scored independently by at least two individuals.

Linkage Analysis

QTLs were mapped using the transformation mean LORR$^{.67}$, Trial 1$^{.54}$, and Trial 2$^{.69}$ (Markel et al. 1996b). Genetic maps were generated using Mapmaker/EXP version 3.0. QTLs were detected in scans using MapMaker/QTL version 1.1. In virtually all cases, the genetic maps produced with the ILS × ISS F_2 mice correlated very well with those published on-line (Mouse Locus Information, http://www.informatics.org/locus.html).

ACKNOWLEDGMENTS

We thank E. Thomas and J. Salazar for careful animal husbandry; G. Hume and J. Costello for technical assistance; A.C. Collins, J.C. DeFries, D. Deitrich, V.G. Erwin, D.W. Fulker, A. Harris, B. Tabakoff, and J. Wehner for helpful advice and manuscript review. This work was supported by grants from the National Institutes of Health (AA-03527, AA-08940), the Denver Veteran's Administration Alcohol Research Center, by a Research Scientist Development Award to T.E.J. (AA00195), and by a training grant for P.D.M. from the National Institute of Mental Health (MH-16880). The mapping data described in this paper will be submitted to the Mouse Genome Database at the Jackson Laboratory.

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Received September 30, 1996; accepted in revised form December 17, 1996.

Confirmation of quantitative trait loci for ethanol sensitivity in long-sleep and short-sleep mice.

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Genome Res. 1997 7: 92-99
Access the most recent version at doi:10.1101/gr.7.2.92