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Genomic screen for QTLs underlying alcohol consumption in the P and NP rat lines

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Abstract. Selective breeding for voluntary alcohol consumption was utilized to establish the alcohol-preferring (P) and alcohol-nonpreferring (NP) rat lines. Inbreeding was initiated after 30 generations of selection and, after 19 generations of inbreeding, 384 F_2 intercross progeny were created to identify quantitative trait loci (QTLs) influencing alcohol consumption. We had reported previously a QTL on Chromosome (Chr) 4; additional markers genotyped on Chr 4 have increased the maximum lod score from 8.6 to 9.2. This QTL acts in an additive fashion and continues to account for approximately 11% of the phenotypic variability. The 95% confidence interval is 12.5 cM and includes the candidate gene, neuropeptide Y. Subsequent to the identification of the QTL on Chr 4, a genome scan was completed to identify additional QTLs influencing alcohol consumption. A lod score of 2.5 was obtained on Chr 3, syntenic to a region previously reported for alcohol preference in mice. Analysis of Chr 8 produced a lod score of 2.2 near the dopamine D2 and serotonin 1b receptors, which have been previously reported as candidate genes for alcohol preference. Evidence for linkage to alcohol consumption was not found on any other chromosome. It therefore appears likely that, in addition to the QTL on Chr 4, multiple loci of small to moderate effect, such as those on Chrs 3 and 8, underlie the difference in alcohol consumption in the P/NP lines.

Introduction

Numerous studies have demonstrated that alcoholism tends to run in families. As Goodwin (1987) has pointed out, 'with almost no exceptions, every family study of alcoholism, regardless of country of origin, has shown much higher rates of alcoholism among the relatives of alcoholics than occurs in the general population'. Twin studies have consistently shown a higher concordance rate for monozygotic than for dizygotic twins, with estimates of heritability ranging from 50% to 60% (Heath et al. 1997). However, 'familial' is not necessarily synonymous with 'hereditary'. In support of a genetic effect influencing alcoholism, rather than a shared environment, the risk for alcoholism is increased even among adopted-away offspring of an alcoholic (Cloninger et al. 1981; Goodwin et al. 1973). Despite this evidence, no genes other than those encoding alcohol and aldehyde dehydrogenase have been clearly shown to influence alcoholism susceptibility (Higuchi et al. 1992; Nakamura et al. 1996; Thomasson et al. 1991). The use of animal models for alcohol-seeking behavior may provide important genetic clues that would improve the efficiency of identifying human genes.

In order for an animal model to provide insight into the genetic mechanism of a human disease, it is necessary that the animal model have properties similar to the human condition. The alcohol-preferring (P) and non-preferring (NP) rat lines were developed at Indiana University for high and low alcohol-seeking behavior through bidirectional selective breeding from a Wistar stock. The P rats have phenotypic characteristics considered to be necessary for an animal model of alcoholism (Cicero 1979) including: 1) high voluntary oral consumption of ethanol leading to high blood alcohol concentrations (50–250 mg%), even in the presence of food and water; 2) consumption of ethanol for its pharmacological effects, rather than its taste, smell, or caloric content, as evidenced by intragastric and intracerebral self-administration of ethanol; 3) operant responding for oral consumption of ethanol at concentrations as high as 30%; and 4) development of metabolic and behavioral tolerance as well as physical dependence when allowed chronic, free-choice alcohol drinking (Li et al. 1993). After the P and NP rats were selectively bred for 30 generations, inbreeding was initiated. At the 19th generation of inbreeding, reciprocal crosses of the inbred P and NP animals generated 384 F_2 progeny.

Lander and Botstein (1989) were among the first to propose the use of inbred animal strains and polymorphic markers to perform interval mapping for identifying quantitative traits. Their methodology exploits the homozygosity of inbred animals at both marker and QTL. By creating F_2 progeny from a cross between inbred strains with quantitative trait measures at opposite extremes of the phenotypic distribution, an F_2 generation with a wide distribution of phenotypic trait values is produced that should be segregating the relevant QTLs. This F_2 generation is then genotyped to identify candidate regions that segregate with the phenotype and hence are likely to be the location of relevant QTLs.

We recently reported a provisional QTL on Chr 4. At the time this QTL was identified, there were not sufficient informative markers to carry out a full genome scan; therefore, informative markers were analyzed separately in the extremely high ($n = 22$) and low ($n = 22$) drinking groups. A cluster of significant markers was located on Chr 4, suggesting that a QTL for alcohol consumption may be located in that region. The entire F_2 sample ($n = 384$) was then genotyped with markers spanning this region, and a lod score of 8.6 was detected (Carr et al. 1998). Comparable evidence for linkage is found in both male and female F_2 progeny. The QTL was shown to act in an additive manner and is estimated to account for 11% of the total phenotypic variability and approximately one-third of the estimated genetic variability. The prepro-neuropeptide

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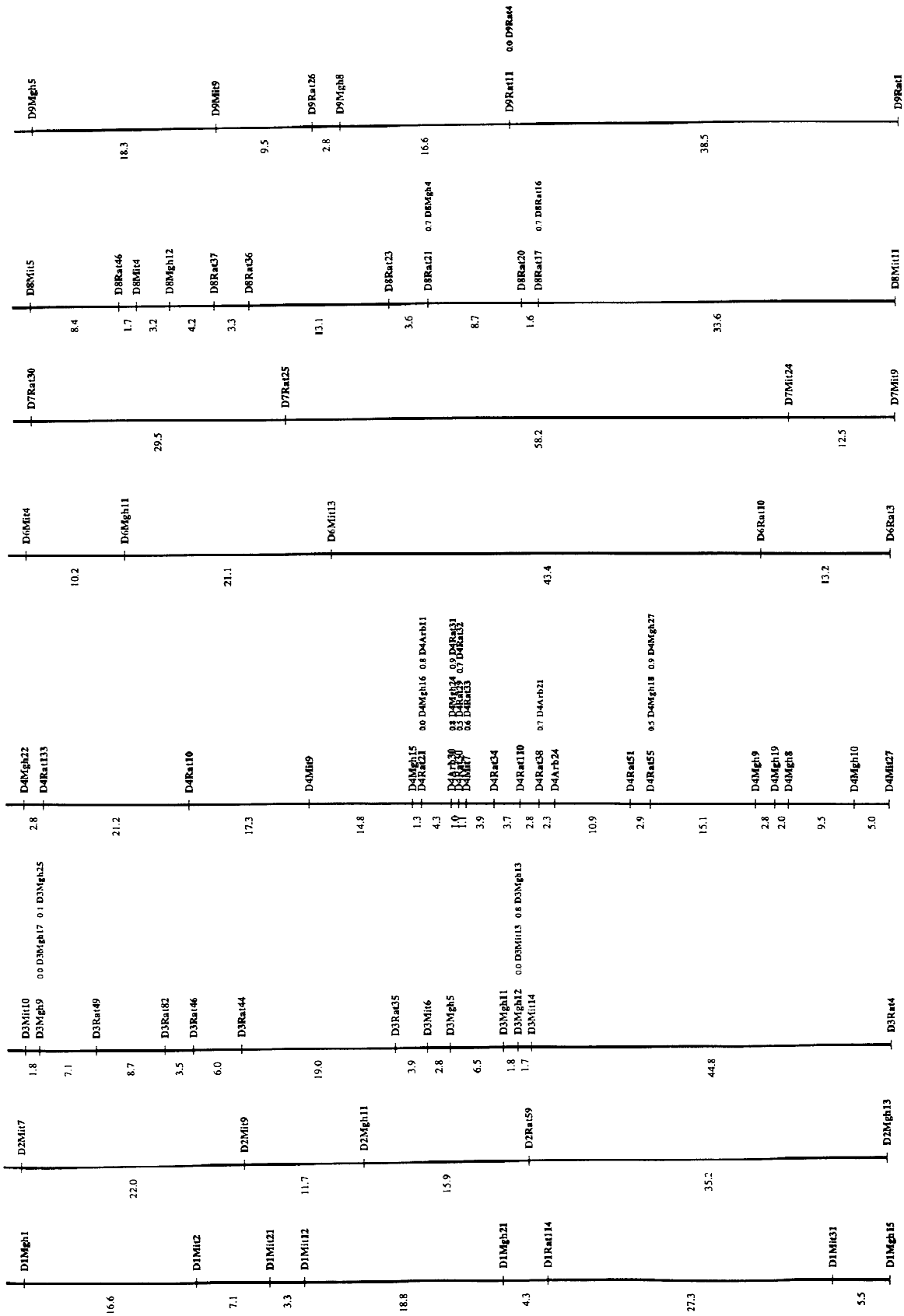

Fig. 1. Genetic linkage map generated using Mapmaker/Exp (Lincoln et al. 1992) for markers informative in the P and NP parental lines.

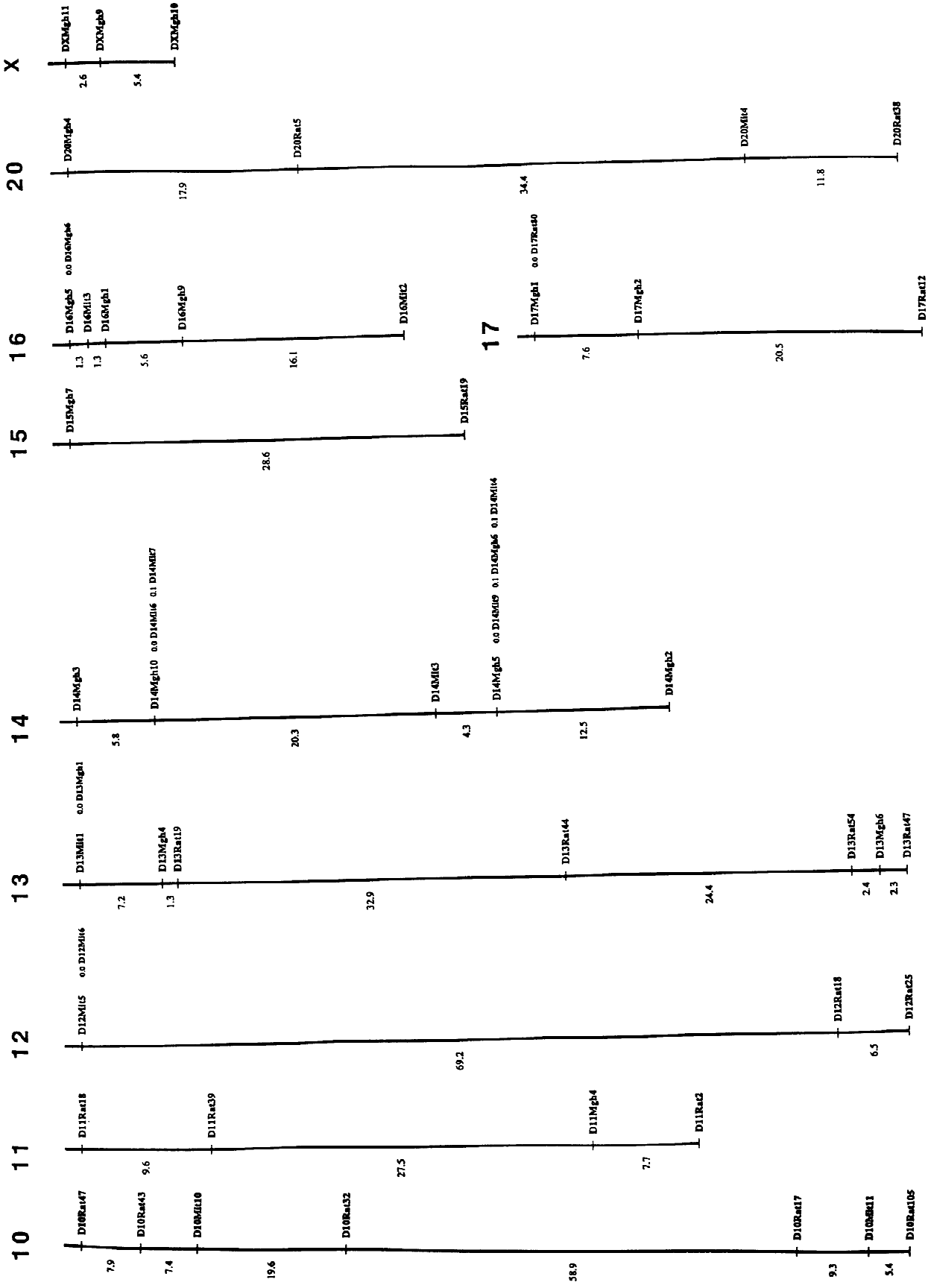

Fig. 1. *Continued.*

Y gene, prepro-*Npy*, is located within this region of rat Chr 4. NPY is an interesting candidate because it has been shown to be anxiolytic and to participate in control of appetitive behavior. We report here the results of a full genome scan that has been completed in the F_2 progeny in order to identify additional QTLs contributing to alcohol preference.

Materials and methods

Alcohol consumption measurement. To measure voluntary alcohol consumption, the rats were individually housed after the onset of puberty and given free access to a 10% ethanol solution (vol/vol) and water for three weeks. Food was available at all times. The amount of alcohol consumed was measured twice a week and the location of the bottles was reversed after each trial to avoid position effects. The drinking scores were adjusted for body weight and an average of six measurements was used in all analyses. The alcohol consumption data (g/kg/day) for all F_2 animals were log transformed because the algorithm used for interval mapping in MAPMAKER/QTL (Lander et al. 1987) assumes a normal distribution of the phenotype. Drinking scores were also determined for alcohol preference, which is the volume of 10% ethanol divided by total fluid volume consumed. The alcohol preference data were normally distributed and therefore not log transformed.

Microsatellite marker genotyping. Genomic DNA was prepared from the spleens of each animal with Puregene™ (Gentra Systems, Inc., Minneapolis, Minn.) according to the manufacturer's protocol. Rat microsatellite markers, selected from those released as of September 1997 by Research Genetics (Huntsville, Ala.), were screened in the parental P and NP animals. Markers were genotyped by methods described previously (Carr et al. 1998). Allele sizes were independently scored by two individuals who were unaware of the animals' drinking scores. Markers polymorphic between the inbred P and NP rats were used to score the F_2 population.

Linkage analysis. Initially, selective genotyping was employed by testing 22 animals at each extreme of the alcohol consumption distribution ($n = 44$; 11% of the distribution) with 100 informative markers released from Research Genetics as of May 1997. Subsequently, with the release of additional markers (June and September 1997), the number of animals selectively genotyped was increased to 46 animals in each tail ($n = 92$, 24% of the distribution) to improve the power for detecting QTLs of smaller effect size. Markers were analyzed individually by the binomial test to identify both additively and dominantly acting QTLs as described by Carr and coworkers (1998). Marker maps were generated with MAPMAKER/EXP (Lincoln et al. 1992), with data from the genotyped F_2 animals. Marker order and distances were compared with those previously published (Brown et al. 1998). Maximum likelihood interval mapping, as implemented in the program MAPMAKER/QTL (Lander et al. 1987), was employed to evaluate evidence for linkage in the selectively genotyped sample. Regions with lod scores greater than 2.0 or binomial test results <0.10 were compared and regions prioritized for additional genotyping in the full sample of 384 F_2 animals.

Prepro-Npy sequence determination. Six sets of sense/antisense oligonucleotide primers were designed on the basis of rat genomic sequences to amplify critical regions of the prepro-*Npy* gene (Larhammar et al. 1987). Primers were as follows: for the −2542 to −2116 region, 5′-CTGCAGTCCTAAGACTTGGGA-3′ (sense) and 5′-GAGCTCTGTCCCACAGGTCAC (antisense); for the promoter, 5′-ACAGATAGGGGCTCGAATCG-3′ (sense) and 5′-GGGAACATCCAAGAGTGAGC-3′ (antisense); for exon 1, 5′-CTCGACGGGGAGAAGTAAAG-3′ (sense) and 5′-GCAAGTTCAAGGGAGTCTAG-3′ (antisense); for exon 2, 5′-CAAAGGCGAGCATTCTCTGC-3′ (sense) and 5′-GTCTCTGGCGTCCAAGATTC-3′ (antisense); for exon 3, 5′-CTCCTATGGTTACTCCAAGC-3′ (sense) and 5′-GGCACCTTAAGCTCTGTAGC-3′ (antisense); for exon 4 and the 3′ flanking region, 5′-GAGAGTCTCACTCTTGTAAG-3′ (sense) and 5′-AGGGCTAACCTAATCCTTTC-3′ (antisense). Amplifications were carried out according to the manufacturer's protocol (Perkin-Elmer, Norwalk, Conn.). All the amplifications were denatured for 1 min at 94°C, annealed at 55°C for 1 min, and extended at 72°C for 1 min. The amplified fragments were cloned with the Original TA Cloning Kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's recommendations. Sequence was determined by dideoxy sequencing, with T7 Sequence version 2.0 according to the manufacturer's protocol (Amersham Life Science, Cleveland, OH).

Results

In total, 153 markers were genotyped in the extreme F_2 animals. Markers were distributed across all chromosomes, although the number of markers per chromosome varied (Fig. 1). Informative markers were not available in all chromosomal regions. Typically, marker order was similar to that previously published (Brown et al. 1998), although intermarker distances varied between the two maps. Differences in marker order and distances may be due to the variable number of F_2 animals genotyped in our study. Multipoint interval mapping was performed on 17 of the 20 rat chromosomes. Linked markers were not available on Chrs 5, 18, and 19, and therefore interval mapping could not be performed for these chromosomes.

Two distinct regions on Chr 3 exhibited lod scores greater than 2.0 in the initial scan. Subsequently, nine markers spanning Chr 3 were genotyped in the full sample of 384 animals, including three new markers in the two regions of interest. In the entire sample, the maximum lod score was 2.5 between the markers *D3Mit10* and *D3Rat49* with the dominant model of QTL action for the alcohol preference phenotype (Fig. 2A), and it does not appear to be gender specific. Similar evidence of linkage to this region was found with the alcohol consumption trait. The second region initially identified in the selectively genotyped animals did not remain significant when the full sample was analyzed.

On Chr 8, a lod score of 2.0 was obtained in the selectively genotyped F_2 animals between the markers *D8Mit5* and *D8Rat46*. Additional genotyping was completed for the three informative markers in the region, as well as a newly identified marker that appeared to be located near the maximum lod score. Linkage analysis using all F_2 animals did not provide additional evidence for linkage (Fig. 2B). A maximum multipoint lod score of 2.2 between the markers *D8Mit5* and *D8Rat46* was obtained under an unconstrained model of QTL action for the alcohol preference phenotype. Similar results were obtained for alcohol consumption.

The binomial test was used to analyze markers that could not be placed unambiguously in marker maps and therefore were not used in the multipoint linkage analyses. Consistent with the lod score analysis, markers on Chrs 3, 4, and 8 had significant binomial test results ($p < 0.05$). While markers on other chromosomes were nominally significant ($p < 0.05$), either the results from adjacent flanking markers were not consistent with linkage or additional informative markers in the chromosomal region were not available.

To narrow the critical interval of a previous QTL finding on Chr 4 (Carr, 1998), we analyzed eight additional markers from this region in the full sample of 384 animals. For alcohol preference, the maximum lod score was 9.2 near the marker *D4Rat34* (Fig. 2C). Similar results were obtained with alcohol consumption (lod = 8.7). After genotyping the additional markers, the 95% confidence interval (Darvasi and Soller 1997) is only 12.5 cM. The QTL mode of action remains consistent with an additive effect and accounts for 11% of the phenotypic variability.

The candidate gene prepro-*Npy* remains within the narrowed support interval. DNAs from the parental animals used to create the F_2 progeny were sequenced to detect differences in the prepro-*Npy* gene (Fig. 3). The prepro-*Npy* gene has four exons, and NPY is encoded by sequences from a portion of exons 2 and 3 (Larhammar et al. 1987). The marker *D4mit7* is located in the second intron, between the exons that encode *Npy*. The four exons and flanking intron sequences as well as 671 bp of the promoter and 360 bp 3′ of the polyadenylation site were sequenced, and no nucleotide differences were detected. Because glucocorticoids ac-

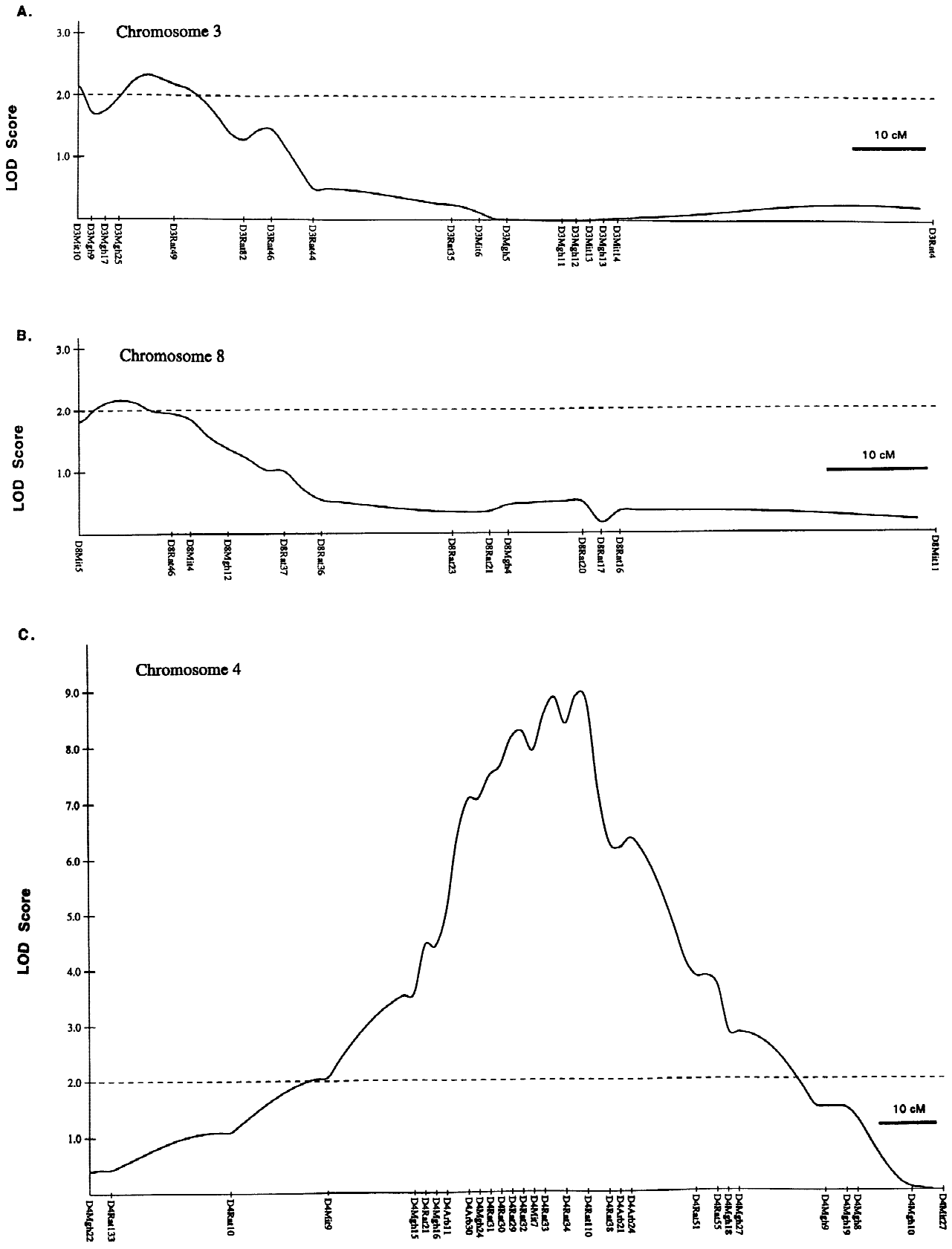

Fig. 2. Multipoint lod scores computed for alcohol preference with the program Mapmaker/QTL (Lander et al. 1987) on: A) Chr 3, dominant model of QTL action; B) Chr 8, unconstrained model of QTL action; and C) Chr 4, additive model of QTL action.

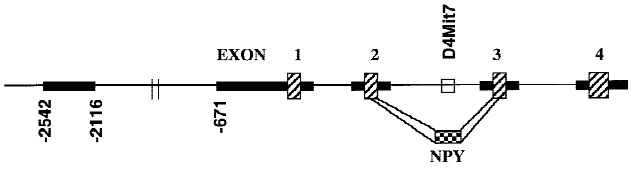

Fig. 3. Schematic of the prepro-*Npy* gene showing regions where nucleotide sequences were compared between the P and NP parental lines. Hatched boxes indicate the exons sequenced, and black boxes indicate the 5′-, 3′-, and intron-regions sequenced. The marker *D4Mit7* and the exons that encode *Npy* are indicated.

tivate the transcription of prepro-*Npy*, three glucocorticoid elements located between −2542 to −2116 (Misaki et al. 1992) were sequenced. No nucleotide differences were detected in this region.

Discussion

An initial genome scan using selective genotyping was performed to identify QTLs for alcohol consumption/preference in the F_2 progeny of a P × NP intercross. Despite initial evidence of linkage to Chrs 3 and 8 from selective genotyping, subsequent genotyping of the full sample of 384 F_2 animals did not provide additional support for a QTL on either chromosome. Neither of these linkage findings meets the statistical significance proposed by Lander and Kruglyak (1995). These two QTL regions are syntenic to mouse chromosomes where QTLs for alcohol preference have been previously identified. A QTL on mouse Chr 9 (syntenic with rat Chr 8) has been provisionally identified in the B × D RI mice (Phillips et al. 1994). In a short-term selection, with F_2 animals derived from the same parental strains, there was suggestive linkage with alcohol preference in this same region (Belknap et al. 1997). Two receptor genes, *Drd2*, encoding the dopamine D2 receptor, and *Htr1b*, encoding the serotonin 1b receptor, appear to be within or near this region of rat Chr 8. Because the rat linkage maps are still being developed, it is not possible to compare syntenic regions between the rat and mouse accurately. The P rats have lower serotonin 1b receptor densities than the NP rats in several limbic regions, including the cingulate cortex, nucleus accumbens, septum, and amygdula (McBride et al. 1997). The P rats also have lower D2 receptor densities in the shell and core subregions of the nucleus accumbens and in the ventral tegmental area (McBride et al. 1993). The differences in densities of these receptors could be a reflection of differences in expression of the respective genes at the transcriptional level. Because gene expression is controlled by a mixture of regulatory proteins that is unique in each cell type, polymorphism in a regulatory region of these genes could result in enhanced or repressed expression of these genes in certain cells in the brain. Thus, the serotonin 1b and dopamine D2 receptors are particularly attractive candidate genes for alcohol preference because dopamine and serotonin neuronal systems have been implicated in the rewarding properties of alcohol and may be important in regulating alcohol drinking behavior in the P and NP rats.

A QTL for alcohol preference has been identified on mouse Chr 2 (Melo et al. 1996; Phillips et al. 1994; Rodriguez et al. 1995) which is syntenic with rat Chr 3. Contrary to our linkage finding in the rat, the QTL identified on Chr 2 by Melo and associates (1996) is male-specific in the mouse. The voltage-gated sodium channel Scn1a is located within this QTL region and is a possible candidate gene.

Previous studies have suggested that alcohol preference/consumption has substantial heritability ($h^2 = 0.35$; Li and McBride 1995) with estimates of four to seven genetic loci (unpublished observation). The chromosome 4 QTL accounts for approximately 11% of the phenotypic variability and perhaps 33% of the genetic variability. Therefore, it is possible that the remaining loci (i.e., regions of Chrs 3 and 8) are of small effect and may require a larger sample size of F_2 animals for detection. An alternative possibility is that additional QTLs may be in regions of the genome in which informative markers have not yet been identified. As new markers are released, these regions will be pursued.

The QTL on Chr 4 is of major effect and remains the highest lod score reported for an alcohol preference phenotype in selected rats. After additional markers were genotyped, resulting in the reduction of the 95% confidence interval, the marker *D4Mit7*, located in the intron of the *Npy* gene, was still within the 12.5-cM critical region. Centrally administered NPY has been shown to be anxiolytic in animal models (Heilig and Widerlov 1995). Mice deficient in *Npy* are hyperexcitable and more susceptible to GABA antagonist-induced seizures. Consistent with a role of NPY in alcohol preference, the P rats are more anxious than the NP rats as measured by the plus-maze test (Stewart et al. 1993) and also have lower levels of NPY in various regions of the brain, including the amygdala, hippocampus and the frontal cortex (Ehlers et al. 1998). Despite little change in food intake (Erickson et al. 1996), the mice deficient in *Npy* consume more alcohol than wild-type mice, suggesting that low levels of NPY result in increased alcohol intake (Thiele et al. 1998). Therefore, based on the anxiolytic properties of the P rats as well as the different levels of NPY in the two lines, we sequenced critical regions of the prepro-*Npy* gene to discern nucleotide differences that might account for the disparate drinking behaviors of the P and NP lines. No mutations were found in the exons and flanking intron sequences or the 5′ and 3′ flanking regions (Fig. 3). Whether polymorphism of the repetitive sequences in the intron of the prepro-*Npy* gene plays a role in its expression or activity remains to be investigated.

Results from both human and animal linkage studies have shown that the genetics of alcoholism and alcohol-related behaviors is even more complex than anticipated. Initially, many hoped that several loci of major effect would underlie these traits and that available methods of analysis would have sufficient power to detect such loci. It appears instead that multiple loci of small to moderate effect likely underlie these phenotypes, and it will be advantageous to consider a larger sample size and additional analysis methods as well as gene-gene and gene-environment interactions to increase the likelihood of QTL detection.

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