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Scanning of five chromosomes for alcohol consumption loci

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Abstract

In our present genetic study to map Quantitative Trait Loci (QTLs) for alcohol-related behaviors, we used 44 B6.C and 36 B6.I inbred congenic Recombinant QTL Introgression (RQI) mouse strains of the b5i7 series carrying genes of BALB/cJ (C) or CXBI (I) origin on C57BL/6ByJ (B6) genetic background. Ethyl alcohol consumption (EAC) was measured in adult males, and chromosomes 1, 2, 3, 9, and 15 were scanned with polymorphic microsatellite markers. In the B6.C set of strains, multiple regression analysis yielded a model with three microsatellite markers, which explained 32% of the genetic variance ($p=0.0006$). The two markers with the highest significance levels in the model, *D1Mit167* and *D2Mit74*, have been mapped to chromosome regions close to the gene opioid receptor kappa 1 (chr. 1) and opioid receptor kappa 3 (chr. 2), respectively. The results of this gene-mapping study suggest that genetic polymorphisms in kappa opioid receptors may contribute to genetic predisposition to voluntary alcohol-drinking behavior.

Keywords: Alcohol; Quantitative Trait Loci (QTLs); Kappa opioid receptor; Mouse strains; Voluntary ethanol consumption; Congenic Recombinant QTL Introgression (RQI) strains; Microsatellite markers

Human alcoholism is a complex disorder, which is partially determined by genetic factors (Slutske et al. 1999). The evolutionary conservation of genes suggests that ongoing gene-mapping efforts to understand alcoholism could be successfully advanced by genetic analysis of animal models. Voluntary ethanol consumption in rodents is an accepted model of alcoholism and is often used to characterize genetic differences in alcohol-related behaviors (McBride & Li, 1998). Gene mapping with congenic strains is an emerging method, which can also provide advanced animal models for mechanism-oriented research (Snell, 1958). Recombinant QTL Introgression (RQI) strains were developed by introgressing dopamine system-related Quantitative Trait Loci (QTLs) onto B6 background by bi-directional selection for the extreme expression of mesencephalic tyrosine hydroxylase activity (TH/MES) with concomitant backcrosses to B6 (Vadasz, 1990; Vadasz et al., 1987). The RQI strains have been bred for more than 30 generations with bxs matings, and offer higher resolution for QTL mapping than Mendelian segregating populations. RQI strains carry passenger genes linked to the introgressed QTLs, and to non-selected, non-linked, randomly introgressed genes, allowing QTL mapping of passenger phenotypes. Because the B6 background strain and the C donor strain have been shown to differ significantly in ethyl alcohol consumption (EAC) in several studies (McClearn & Rodgers, 1959; Belknap et al., 1993; Le et al., 1994; Vadasz et al., 1996a), we studied two measures of alcohol-related behaviors: consumption of 12% (v/v) unsweetened ethyl alcohol solution (EAC, g/kg per day), and total liquid consumption (TLC, g/kg per day). EAC reflects the propensity to drink ethanol and TLC reflects the total water requirement of the subjects. We concentrated on the mapping of five mouse chromosomes, because previous studies suggested that these chromosomes may carry loci for alcohol-related behaviors (Melo et al., 1996; Vadasz et al., 1996a, 2000; Buck, 1998; Tarantino et al., 1998).

1. Methods

1.1. Animals

RQI strains were constructed as described (Vadasz, 1990; Vadasz et al., 1994a,b, 1996b, 1998). Briefly, QTLs that are responsible for the continuous variation of mesencephalic tyrosine hydroxylase activity (TH/MES) were introgressed onto the C57BL/6By (B6) strain background

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from BALB/cJ (C) and CXBI (I) donor strains with high and low TH/MES, respectively. I is a recombinant inbred strain carrying B6 and C genes (Bailey, 1981). B6 served as the background strain, because its TH/MES was intermediate between those of the donor strains and it had already been used as a background strain for numerous congenic lines. Two types of F2s, B6XC and B6XI, were produced. In each type replicates (alpha and beta) were created by equal division of each F2 litter. Thus, four closed lines were established. In each line, at least 45 F2 males were tested for the phenotype, and 15 were selected for the first backcross to B6 females. Then, at least 45 backcross1 (b1i0) male offspring was tested, and 15 males were selected and intercrossed with non-littermate females resulting in the b1i1 generation. The QTL transfer was carried out in two directions by backcross–intercross cycles with concomitant selection for the extreme high and low expressions of TH/MES in replicates, resulting in four QTL introgression lines. In the B6.C and B6.I QTL Introgression (QI) lines, the top and bottom one-third of each generation were selected, respectively (Vadasz et al., 1994a,b). These steps were repeated for five cycles [with some variation between cycles: double intercrosses were made in two cycles because of diminishing difference from the background; a higher number of males were tested in later cycles, etc. (see Vadasz et al., 1994a,b, 1998)] As to common ancestors of b5i7 mice, in each of the four closed QI lines, because of the approximately 33% selection (in addition to occasional infertility, death, etc.), a part of the contribution of the original 15 F2 genomes must have been eliminated during the 13 generations of selection, and it was expected that the selectively favored introgressed part of the donor genome would not represent equally the genome of each of the original 15 F2 males. However, assuming an appropriately large population, we expected that the introgression of the non-selected, non-linked donor genome would be random. Considering just one closed QI line, the co-ancestors of the eventually derived RQI strains were 15 F2 males and the isogenic common B6 females. The recombinant chromosome sets produced by the 15 F2 males were carried by b1i0 female and male offspring in 15 families (in about 90 descendants). It was expected that the selected 15 b1i0 males would not represent all 15 F2 males because of the selection pressure applied. These males were mated with 15 non-phenotyped b1i0 females who carried approximately random chromosome segments of any of the 15 F2 males because they were chosen by the following rules: (1) no littermate mating was allowed, and (2) females were chosen "random" (though they were not randomized statistically). The use of the random-selected (non-littermate) b1i0 females ensured that the original donor chromosome segments of the 15 F2 males would become redistributed in the b1i0 females and be transmitted to the b1i1 generation, while the use of the selected b10i males ensured that a part of the original donor chromosome segments of the 15 F2 males would become eliminated by selection. In the course of the QI line development, there was a repeated redistribution and selective favoring of a portion of the donor genome in each intercross (seven altogether), and the current male haplotype was replicated in females of each backcross generation, which made possible the transmission of the selected gene pool of the males in the next intercross. This mechanism suggests that all the 15 F2 males are common ancestors, as a pool, of the b5i7 individuals. The QTL introgression phase was followed by initiation of bxs mating for at least 30 generations. The bxs regime started with 36 founder mating pairs (selected from about 60 phenotyped b5i7 males and a pool of b5i7 non-littermate females) in each of the four closed sets (B6.C-Alpha, B6.C-Beta, B6.I-Alpha, B6.I-Beta). Each RQI strain was derived from a different pair of b5i7 parents, with the exception of the following cases: (1) C5A15 and C5A16, (2) C5A8 and C5A9, (3) C5B1, C5B2, and C5B3, (4) C5B5 and C5B6, (5) I5A14 and I5A15, (6) I5B11 and I5B12, (7) I5B6 and I5B7 (Table 1). Strains in each of the above seven cases were derived from the same parental pair, i.e., seven b5i7 parental pairs gave rise to 15 RQI strains. These extra strains and several sublines were created with the intent to counterbalance the expected loss of strains caused by inbreeding and other factors. After about 30 generations of bxs mating, strains derived from a common b5i7 mating pair often showed significant differences (Table 1), and did not show significant similarity in their marker genotypes. Therefore, we decided to use these extra ("less-independently derived") eight strains in the regression analysis without correction.

For the alcohol experiment 9–11-week-old mice were used and had been in the study room for at least 1 week prior to the 3% trial. All procedures followed guidelines consistent with those developed by the National Institute of Health and the Institutional Animal Care and Use Committee.

1.2. Nomenclature

We use abbreviated RQI strain names. For example, the full name of one of the RQI strains is B6.Cb5i7-β3/Vad, and it is abbreviated as C5B3. The first letter, C (or I), stands for the donor strain name; 5 (or 4) designates the backcross–intercross series b5i7 (or b4i5); B (or A) indicates the replicate line β (or α); and the last character, 3, is the identification number of the strain (the numbers range from 1 to 34 in each replicate line). The RQI strains are referred to as "congenic" in the same sense that "congenic" is used for the Recombinant Congenic (RC) series (Demant & Hart, 1986).

1.3. Behavioral testing

The capacity of the testing system allowed us to carry out ethanol-preference tests in batches of 70 subjects. Depending on the availability and total number of animals

Table 1
Alcohol and total liquid consumption

Strain	Alcohol[a]	*n*	Total liquid[a]	*n*
B6	7.82 ± 3.69	85	176.96 ± 24.24	76
C	0.12 ± 0.16	75	176.60 ± 27.79	76
C5A1	8.02 ± 4.14	16	226.85 ± 42.59	16
C5A12	9.80 ± 3.21	18	216.56 ± 27.84	18
C5A13	5.78 ± 3.59	17	200.52 ± 34.29	17
C5A15	5.20 ± 2.03	19	171.35 ± 15.74	20
C5A16	4.51 ± 2.81	13	180.08 ± 28.28	13
C5A17	5.68 ± 2.87	17	175.66 ± 23.36	17
C5A18	5.59 ± 3.98	17	210.80 ± 39.68	17
C5A19	6.11 ± 3.77	14	188.65 ± 40.46	14
C5A24	7.21 ± 3.83	11	281.19 ± 82.60	11
C5A26	6.51 ± 3.62	16	190.69 ± 45.39	16
C5A3	2.89 ± 3.02	20	168.61 ± 30.91	20
C5A32	8.70 ± 4.58	20	175.93 ± 28.61	20
C5A32A	7.54 ± 3.38	17	190.89 ± 20.00	16
C5A34	5.74 ± 4.67	17	212.03 ± 44.56	17
C5A35	6.02 ± 3.02	21	248.58 ± 80.07	18
C5A4	7.91 ± 3.52	14	185.96 ± 16.35	14
C5A5	7.11 ± 3.87	23	184.43 ± 21.10	23
C5A6	7.89 ± 3.82	17	171.01 ± 23.33	17
C5A7	9.78 ± 4.40	17	182.69 ± 28.22	17
C5A8	9.27 ± 3.72	24	164.80 ± 23.57	24
C5A9	6.73 ± 4.43	11	174.60 ± 26.76	11
C5B1	5.88 ± 2.37	16	176.81 ± 23.48	14
C5B10	8.93 ± 3.38	13	200.24 ± 24.68	13
C5B12	7.21 ± 4.08	20	194.00 ± 26.66	20
C5B13	7.37 ± 3.61	17	198.15 ± 19.30	17
C5B15	3.95 ± 2.50	20	194.26 ± 29.34	20
C5B16	5.40 ± 4.36	20	201.40 ± 28.18	20
C5B19	5.00 ± 2.65	15	176.75 ± 23.41	15
C5B2	3.98 ± 2.32	18	157.62 ± 21.10	18
C5B20	8.42 ± 3.76	18	174.02 ± 13.99	18
C5B23	4.88 ± 3.66	24	166.04 ± 24.66	23
C5B25	5.20 ± 3.10	16	196.34 ± 18.29	16
C5B26	8.11 ± 2.95	20	167.82 ± 28.30	20
C5B27	5.62 ± 2.72	24	218.35 ± 31.54	23
C5B28	8.89 ± 3.78	17	185.43 ± 28.57	17
C5B3	2.70 ± 1.72	14	183.83 ± 28.03	15
C5B30	6.51 ± 3.69	16	204.68 ± 30.35	16
C5B31	7.79 ± 3.55	17	229.22 ± 37.78	16
C5B33	6.83 ± 3.24	14	227.56 ± 44.94	14
C5B34	8.20 ± 4.22	17	185.74 ± 36.07	17
C5B4	8.96 ± 4.78	12	185.81 ± 30.83	11
C5B5	5.25 ± 3.04	18	209.33 ± 17.48	18
C5B6	8.50 ± 3.60	16	199.62 ± 21.91	15
C5B9	4.49 ± 3.50	8	212.75 ± 24.48	8
I	1.16 ± 1.29	74	230.63 ± 37.55	73
I5A10	5.59 ± 4.44	13	193.63 ± 30.73	13
I5A11	5.21 ± 3.65	24	182.35 ± 34.84	24
I5A12	7.24 ± 2.19	16	186.89 ± 41.87	16
I5A14	8.63 ± 3.43	10	223.15 ± 29.71	10
I5A15	8.61 ± 3.39	22	171.07 ± 21.22	22
I5A16	6.47 ± 3.51	16	196.56 ± 50.00	16
I5A16at	7.20 ± 3.90	24	168.57 ± 21.14	21
I5A19	4.93 ± 2.98	17	180.86 ± 34.52	17
I5A22	7.31 ± 2.59	18	226.57 ± 35.56	18
I5A25	4.85 ± 2.53	20	216.45 ± 37.78	20
I5A26A	6.22 ± 2.90	15	214.19 ± 55.53	15
I5A27	8.09 ± 3.87	20	193.30 ± 30.15	20
I5A31	5.23 ± 2.22	17	200.34 ± 29.77	19
I5A33	7.68 ± 4.70	16	188.38 ± 22.36	15
I5A4	5.33 ± 3.29	21	221.86 ± 33.54	21
I5A5	6.44 ± 2.45	11	191.50 ± 14.06	11
I5A8	7.28 ± 2.44	17	202.92 ± 19.39	17
I5A9	6.39 ± 3.07	17	190.91 ± 23.35	15
I5B1	5.93 ± 4.06	16	232.99 ± 49.87	16
I5B11	4.88 ± 2.90	17	154.77 ± 34.95	16
I5B12	5.12 ± 3.50	22	211.98 ± 49.69	22
I5B14	4.01 ± 1.77	17	170.41 ± 35.09	17
I5B15	5.77 ± 2.85	13	231.20 ± 32.15	13
I5B16	7.56 ± 4.43	20	172.74 ± 15.30	17
I5B19	7.05 ± 3.68	13	181.20 ± 19.44	13
I5B1A	6.89 ± 3.81	17	214.19 ± 26.69	17
I5B22	9.16 ± 2.82	24	172.15 ± 20.79	24
I5B23	6.83 ± 3.79	19	175.73 ± 16.00	19
I5B24	5.54 ± 3.51	17	195.73 ± 23.31	17
I5B25A	11.89 ± 2.33	22	165.62 ± 20.51	22
I5B27	5.11 ± 2.51	16	193.23 ± 30.09	16
I5B31	9.07 ± 3.50	21	181.52 ± 25.85	20
I5B33	6.10 ± 3.24	16	175.78 ± 24.42	16
I5B34	4.59 ± 2.78	20	173.31 ± 25.12	20
I5B6	9.47 ± 3.21	16	215.79 ± 58.46	16
I5B7A	7.18 ± 2.88	17	214.68 ± 46.96	17

For each animal the results of three consecutive 3-day-long trials were averaged.

[a] Results are expressed as grams per kilogram per day mean values ± SD with number of animals (*n*).

tested, in each batch, three littermate males of available RQI strains were tested, along with three males of each of the progenitor strains. The latter served as standard reference throughout the phenotyping to assess inter-assay variability. We used a slightly modified "two-bottle choice" paradigm with escalating ethanol concentration and with limited social isolation (Vadasz et al., 2000) to measure ethanol preference. We modified the method by increasing the number of the 3-day-long trials of consumption of 12% ethyl alcohol from two to three trials.

1.4. Genotyping

Genomic DNA was prepared from tail tips (Miller et al., 1988) of stem-line animals (in most cases), and samples from one male and one female were pooled. Markers polymorphic for B6 and C strains were chosen as PCR primers with information from the Mouse Genome Database (Mouse Genome Informatics, The Jackson Laboratory). In order to analyze PCR products with an ABI 310 Genetic Analyzer, dye (FAM, TET, or HEX)-labeled microsatellite markers were custom-synthesized by Integrated DNA Technologies, Coralville, IA, or purchased from Research Genetics, Huntsville, AL. Multiplex PCR was performed in 10 μl of PCR buffer containing 1.5 mM $MgCl_2$, 50 ng of template DNA, six pairs of dye-labeled primers (2 FAM, 2 TET, and 2 HEX-labeled, each 240 nM), dNTPs (each 200 μM), and Taq DNA polymerase (0.5 U, AmpliTaq Gold, PE Applied Biosystems, Foster City, CA). Reactions were performed in HI-TEMP 96 microplates (Techne, Princeton, NJ) using MW-2 Dri-

Plate Cycler (Techne). The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 33 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min, then by a final extension period at 72°C for 7 min. One percent of the PCR products was mixed with 12 μl of formamide and 0.5 μl of GENESCAN-500 (TAMRA) Size

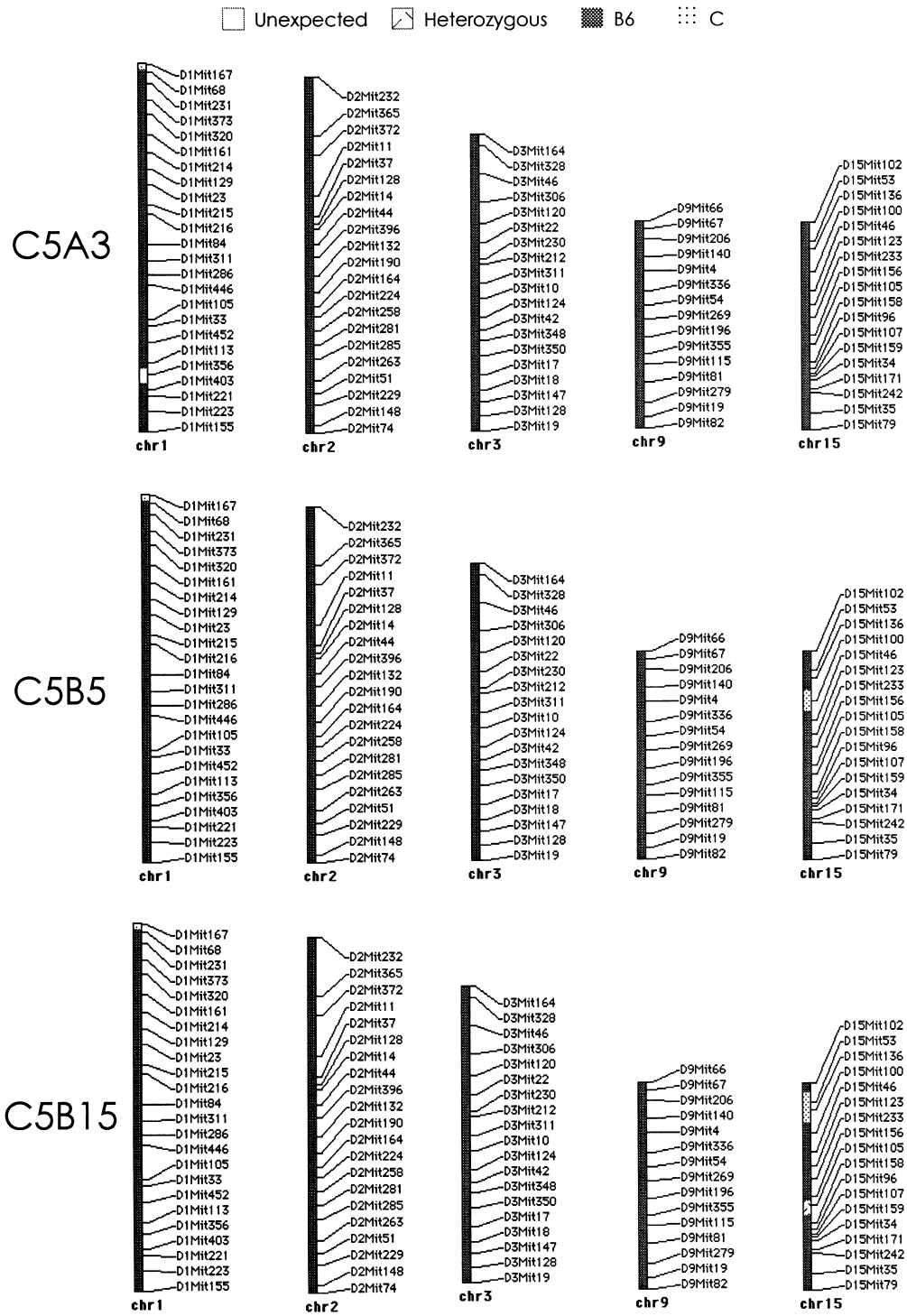

Fig. 1. Graphical genotypes of B6.C RQI strains showing the distribution of 97 microsatellite markers used in QTL mapping with 80 RQI strains. The displayed congenic RQI strains carry C-type *D1Mit167* alleles, and their mean EAC is significantly lower than the mean EAC score in RQI strains carrying two copies of the B6-type allele of *D1Mit167*. Dark and light stippled chromosome regions represent background B6 and donor C genotypes, respectively; empty (white) areas indicate an unexpected marker type that is possibly due to mutation. In C5B15, *D15Mit105* was heterozygous. However, recent genotyping data (not shown) suggest that most individuals in C5B15 are homozygous B6-type at that locus. Marker locations correspond to the Chromosome Committee positions.

Standard (PE Applied Biosystems), denatured for 3 min on a 95°C heating block, and applied on the capillary (47 cm×5 μm) of an ABI 310 Genetic Analyzer.

1.5. Mapping

Mapping was performed with the QGene program package (Nelson, 1997). In permutation analysis, for both EAC and TLC, at each locus, the marker data were randomly shuffled 1000 times, redoing a regression with every shuffle and recording the fit statistic. For each locus, the 95th percentile of the F values for that set of shuffles was determined and compared to the phenotype-marker regression statistic. For multiple regression analysis, models were created by including markers (factors) with F scores higher than the 95th percentile. The reported p values refer to point-wise significance levels, unless stated otherwise.

1.6. Estimation of donor chromosome segment size

When two or more adjacent donor loci were observed in a chromosome region, the interval between the two outermost donor loci was calculated, and half of the intervals between the outermost donor loci and the flanking background loci were calculated on each side, and added to the interval between the outermost donor loci. If the donor locus was flanked by the centromere or telomere, the full-length of that region was used to estimate the maximum segment length. The estimate of the number of genes on a segment was based on the assumption that 70,000 murine genes are distributed on the chromosomes with a total length of 1600 cM.

2. Results

EAC and TLC scores were obtained for 1743 male mice representing three progenitor strains and 80 RQI strains. In Table 1, we summarized the phenotypic differences between the progenitor and congenic RQI strains. The donor C and I mice showed significantly lower EAC than B6 background mice. All RQI strains exhibited higher EAC than C or I, and several RQI strains showed higher or lower EAC than the background strain. TLC was not significantly different in B6 and C mice; however, I mice showed higher TLC than the other two progenitor strains. One-way ANOVA indicated significant strain-dependent variation of both phenotypes in the 80 RQI strains ($p<0.001$). Comparison of the two different introgression types B6.C and B6.I showed that the mean EAC and TLC scores were not significantly different ($p>0.05$, indepen-

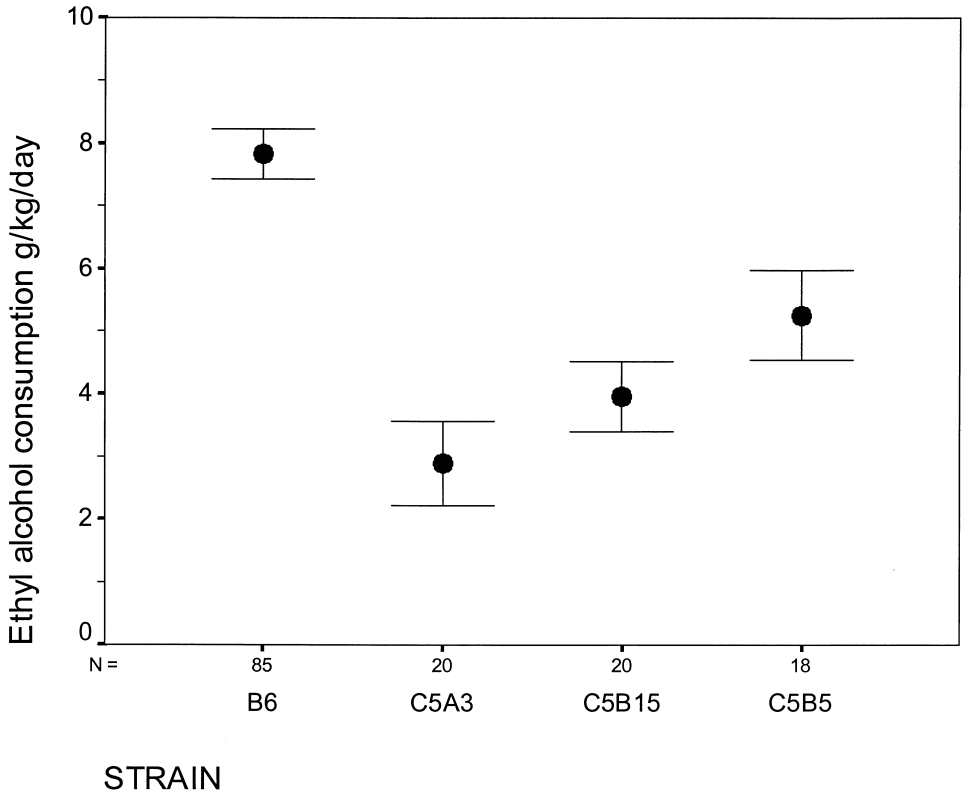

Fig. 2. EAC in progenitor and RQI strains. The background strain B6 shows significantly higher EAC than the congenic RQI strains (one-way ANOVA, $p<0.001$; followed by Tukey's HSD, $p<0.05$). The three congenic B6.C RQI strains (C5A3, C5B15, C5B5) carry two copies of the C-type allele of *D1Mit167* derived from the C donor strain. Data are shown as mean±1 SEM.

Table 2
Results of multiple regression analysis of EAC (g/kg per day)

	Class means			QTL effect size	p[a]
Marker	AA	aa	Aa		
D1Mit221	6.52	9.27	8.96	−1.37	0.0476
D1Mit167	6.83	4.03	–	1.4	0.0035
D2Mit74	6.73	2.70	–	2.01	0.0098

[a] The presented p values represent the significance of the partial regression coefficients. The significance of the model consisting of the three markers is $p=0.0006$.

dent samples t test). Both the genetic and phenotypic correlations between EAC and TLC were low in the 80 RQI strains ($r=0.02$, $p>0.05$ and -0.13, $p<0.05$), indicating that the variables are genetically independent.

Chromosome scanning of 44 B6.C and 36 B6.I RQI strains and their progenitors was performed with 97 microsatellite markers, providing an average marker spacing of 4.91 cM (Fig. 1). The marker–phenotype association was analyzed separately in the two different introgression types B6.C and B6.I with the QGene program by permutation analysis and multiple regression. Permutation analysis in the B6.C set of strains for EAC detected four statistically significant markers: *D1Mit167* (6.5 cM, $p<0.01$), *D1Mit221* (102 cM, $p<0.05$), *D2Mit148* (105 cM, $p<0.01$), and *D2Mit74* (107 cM, $p<0.01$). For chrs. 9 and 15, markers with the highest correlation were *D9Mit54* (39 cM) and *D15Mit105* (42 cM); however, their F values were smaller than the 95th percentile ($p<0.1$). For TLC no significant marker was found.

Donor chromosome segments carrying homozygous C-type *D1Mit167* were detected in three RQI strains, C5A3, C5B5, and C5B15 (Figs. 1 and 2). When EAC was compared between the B6 background strain and three congenic RQI strains bearing two copies of the allele derived from the C donor strain, one-way ANOVA indicated highly significant strain-dependent variation ($F_{3,139}=16.31$; $p<0.0001$). Tukey's HSD (using harmonic mean sample size=23.91) yielded two non-overlapping homogeneous subsets of strains at $\alpha=0.05$ level. Subset 1 comprised C5A3, C5B15, and C5B5, whereas subset 2 contained B6 alone, suggesting that allelic status at the microsatellite marker *D1Mit167* was associated with EAC. In each of the three strains, the homozygous C-type *D1Mit167* was flanked distally by the B6-type *D1Mit68* (9 cM), while the genotype of the markers between *D1Mit167* and the centromere was not known. In our estimate, the length of the donor segment was less than 8.25 cM (see Section 1). We tried to establish the segment size more precisely by genotyping additional markers in these strains. *D1Mit64* (5 cM) turned out to be non-polymorphic, while *D1Mit65* (8.4 cM) were B6-type, yielding a new estimated segment size of less than 7.5 cM. This segment size is close to the average donor segment length in RQI strains of the b5i7 series (6.63 cM, SD=3.87). The estimated donor segment sizes for the other two regions, identified by *D1Mit221* (102 cM) and the linked *D2Mit148* (105 cM) and *D2Mit74* (107 cM), were 3.15 and 12 cM. In the B6.I set of RQI strains, no significant marker–phenotype association was detected ($p>0.05$).

Of the 97 markers used for scanning the five chromosomes, the genotype of 44 markers was B6/B6 in all tested

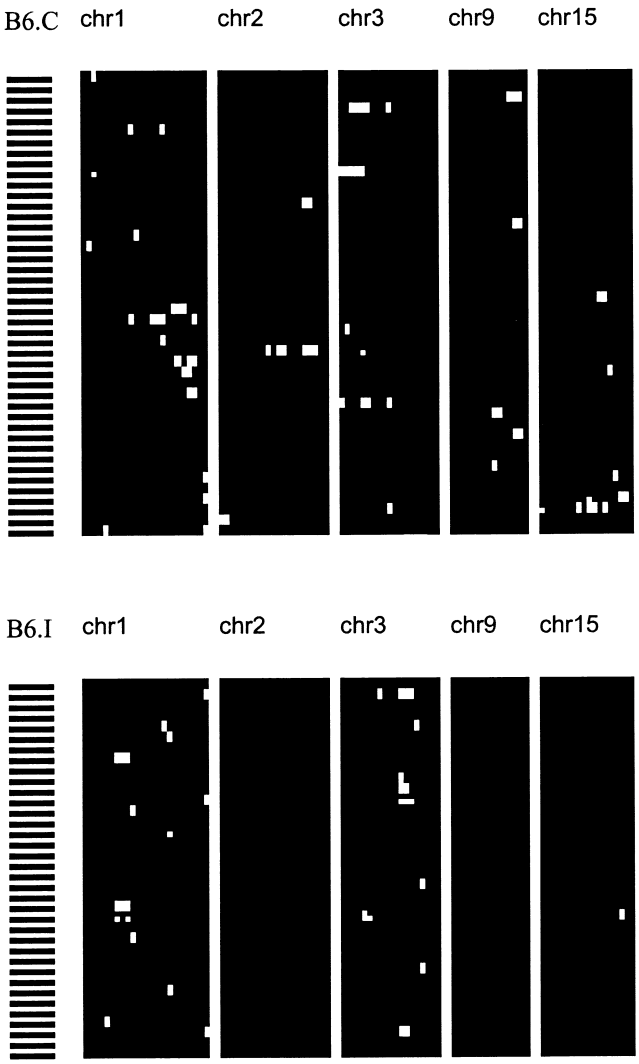

Fig. 3. Chromosomal distribution of donor C-type genes (white rectangles) on C57BL/6By background (black rectangles) in the B6.C (upper panel) and B6.I (lower panel) sets. Results of the marker scanning are graphically summarized for each RQI strain. Contiguous horizontal lines under a chromosome number represent a chromosome for a given strain with its centromere on the right end. The strains, represented by the short horizontal bars on the left of the figure under B6.C, are in the following order, from top to bottom: A8, A7, A32, B28, B26, B20, A6, B10, B4, A12, B34, B6, A4, A5, A32A, A9, B12, B13, A26, A1, B31, A19, B1, A15, A17, B30, A34, A13, B33, B19, B23, A24, A18, A35, A16, B25, B16, B2, B5, B27, B15, B9, B3, A3. Likewise, for the B6.I set the strains are in the following order: B25A, B22, A15, B31, B6, A27, A33, A16at, A12, A14, B19, B23, B16, B33, A8, B7A, A5, B11, A22, B1A, A26A, A16, A9, A11, A31, B24, A19, B1, B27, A10, B34, B12, B15, B14, A4, A25 ("C5" (for the B6.C set) and "I5" (for the B6.I set) are omitted from the above abbreviated RQI strain names). The RQI strains of the B6.I set do not show the presence of C-type donor segments in chrs. 2 and 9 (see text for further information).

congenic RQI strains. For multiple regression analysis, we selected those markers whose F scores were higher than the 95th percentile of F scores obtained in permutation analysis after 1000 shuffles. *D1Mit167* and *D2Mit148* had F scores higher than the 99th percentile, while the F score for *D1Mit221* was higher than the 95th percentile. The B6.C EAC data with the above three factors yielded a model that explained 32% of the genetic variance (adjusted R^2; $F_{3,40}=7.21$; $p=0.0006$; Table 2). In the model, the partial regression coefficients of the markers were significant: $p=0.0035$ (*D1Mit167*), $p=0.0098$ (*D2Mit74*), and $p=0.0476$ (*D1Mit221*).

Because the B6.C and B6.I sets of RQI strains represent two independently developed populations of inbred strains with different donor strains (BALB/cJ vs. CXBI) and different direction of genetic selection for TH/MES (high vs. low), we used this opportunity to perform a partial comparison (restricted to chrs. 1, 2, 3, 9, and 15) of the genomic compositions of these two different introgression types (Fig. 3). The comparison of the B6.C and B6.I sets showed that the B6.C set carried several donor segments on chrs. 2 and 9, but no donor segments were present in the B6.I set on these two chromosomes.

3. Discussion

On chr. 1, at a distance of 1 cM from *D1Mit167*, we found *Oprk1* (opioid receptor kappa 1, Yasuda et al., 1993), which maps at 5.5 cM from the centromere. There is strong evidence demonstrating that the opioid system plays a significant role in alcoholism (Koob et al., 1998). Several recent studies compared the alcohol-preferring B6 strain and the alcohol-non-preferring DBA/2 strain for expression and content of kappa-opioid receptors and their dynorphin ligands. The DBA/2 strain presented a significantly higher content of kappa opioid receptor binding sites and prodynorphin mRNA in the nucleus accumbens (Jamensky & Gianoulakis, 1997), and higher expression of kappa opioid receptor mRNA in other limbic areas (Winkler & Spanagel, 1998). Enhanced stimulation of the kappa opioid receptor in the nucleus accumbens has been associated with decreased dopamine release and aversive states (DiChiara & Imperato, 1988). Kappa receptors reside on axons of dopaminergic A10 cells (Svingos et al., 1999), which arise from the midbrain to innervate the nucleus accumbens, a structure prominently involved in the brain reward mechanisms and alcohol preference. Previous mapping studies by others showed associations between alcohol preference and a region of chr. 1 including *Odc-rs10* (13 cM) and *D1Rti2* (29.8 cM) (Rodriguez et al., 1995). Recently, this provisional QTL was confirmed (Whatley et al., 1999) and a QTL (*Alcp5*) was suggested for alcohol consumption on chr. 1 somewhere between the centromere and 16 cM (*D1Mit169*). Interestingly, an earlier report assigning an alcohol consumption QTL (*Alcp4*) to this region found linkage with *D1Mit295* (8.3 cM) in females, and concluded that this locus is female-specific (Peirce et al., 1998). Although the probable ranges for the location of the proposed QTLs *Alcp4* and *Alcp5* are overlapping, it was argued (Whatley et al., 1998) that *Alcp5*, associated with *D1Mit65* (8.4 cM), is not the same QTL as *Alcp4*, because the latter is female-specific. Our studies were done on males, and defining the QTL-harboring interval, we excluded regions distal from 8.3 cM. Future testing of females will answer the question of whether this chr. 1 QTL is sex-specific in our congenic strains.

Identification of a QTL for EAC on a small chromosome region in congenic RQI strains in the proximity of *Oprk1*, the above supporting neurochemical evidence for a role of opioid receptors in alcohol consumption, and the results of previous mapping studies raise the possibility that the QTL detected in this study is identical with *Oprk1*.

Another donor region, detected on chr. 2 (Table 2), may deserve further attention. *D2Mit74*, which resides at 107 cM, is close to *Oprl* (opioid receptor-like, 110 cM) (Mollereau et al., 1994). It was claimed that this receptor is identical to a form designated as kappa 3 receptor (Pan et al., 1996). In line with our results, a study with AXB/BXA RI strains also showed significant association between *D2Mit74* and alcohol preference in both sexes (Gill et al., 1996).

The third provisional QTL in the multiple regression model was marked by *D1Mit221* (102 cM), barely reaching the 5% level of significance. In contrast to the kappa opioid receptors, the C-type alleles appeared to confer an increase in alcohol consumption. A member of the transforming growth factor beta (TGFB) superfamily, *Tgfb2*, has been mapped at 101.5 cM. *Tgfb2* has neurotrophic and neuroprotective effects on dopaminergic neurons (Sullivan et al., 1998). Because neurotrophic factors can induce a dopaminergic phenotype and increase locomotor activity (Lapchak et al., 1997; Choi-Lundberg et al., 1998), *Tgfb2* may affect the development of the dopaminergic system, influencing neurotransmission and reward. However, this QTL is a weaker candidate for affecting alcohol consumption in mice.

Criteria for significance were recommended for various designs (Lander & Schork, 1994; Lander & Kruglyak, 1995; Belknap et al., 1997). For RI lines, 1 degree of freedom, two-sided test, a p value of 2×10^{-5} (lod score 3.9) (Lander & Schork, 1994), and for selection lines (S_4 generation) (lod score 3.8) (Belknap et al., 1997), were suggested. For alcohol consumption the significance of the multiple regression model with three suggestive QTLs was 6×10^{-4}, not attaining the above criteria for significant linkage. However, the properties of the RQI design are rather different (its development involves backcrosses, intercrosses, selection, and a final RI-type breeding scheme), and the significance criteria for RQI QTL results remain to be worked out. Two other sources of concern are that in this study, we scanned only 5 of the 19 autosomes, and the average marker density

is >4 cM, with the largest gap of 17 cM. This suggests that (1) there may be additional QTLs located on the non-scanned chromosomes, (2) statistical analysis of a full genome scan may shed a different light on the current results, and (3) we may have missed QTLs located on the scanned chromosomes because some of the introgressed segments could be shorter than our marker spacing, thus leaving donor segments undetected. As a part of our general strategy, we plan to characterize the full genome of all RQI strains at a higher resolution, and verify significant strain-comparison-based mapping results in segregating generations (Fijneman et al., 1996; Moen et al., 1996).

Generalizability of the results is another issue. In most previous studies, C57BL/6J and DBA/2J strains were used, whereas our strains were derived from C57BL/6By and BALB/cJ, raising the possibility that the differences between C57BL/6J and DBA/2J on the one hand, and C57BL/6By and BALB/cJ on the other, may involve some of the same and some different QTLs that affect alcohol consumption.

The designs used for QTL mapping in the past decade differ in many features including the QTL effect size. QTL effect size depends on the type of experimental design. QTL effect size reflects gene effect size, which is defined by the half of the allele class mean difference. Currently, QTLs are usually detected by markers located on the same chromosome. The distance (cM) between the gene underlying the QTL and the marker is critical in the analysis of mapping data. Accordingly, we have to distinguish marker effect size (or apparent QTL effect size; E_m), from real QTL effect size (E_{QTL}). The marker effect size, which is defined by the half of the marker class mean difference, is a composite of the real QTL effect size and the single generation recombination frequency, c in Eq. (1):

$$E_m = E_{QTL}(1 - 2c). \quad (1)$$

If multiple rounds of recombination are involved in the development of the design, markers in the F_t generation will show an expansion of the genetic map relative to an F_2. Designs with higher frequency of recombination, such as AIL (Darvasi & Soller, 1995) and RQI (Vadasz, 1990) are advocated because they offer higher precision for estimates of QTL position, but, these constructs are also expected to show reduced QTL effect size (E_m). However, the RQI design relies on congenic inbred strains; thus, the real QTL effect size (E_{QTL}) can also be estimated by comparing a congenic RQI strain, which carries a confirmed QTL, to the background B6 strain. It is expected that the real QTL effect size is the same as the marker effect size, because two isogeneic strains are compared, and c is not part of the equation anymore. This expectation is supported by our results. For example, we found in the B6.C RQI b5i7 series of strains for *D1Mit167*, AA=6.83, aa=4.03, and the marker effect size (E_m) is 1.4. In congenic strain C5A3 (carrier of aa genotype for *D1Mit167*) and background partner strain B6 (AA genotype), alcohol consumption was 2.89 and 7.82, respectively. Assuming that the provisional *D1Mit167* marked QTL will be confirmed and it is the only QTL carried by C5A3, the congenic-vs.-background effect size (E_m*) is 2.46. Also, considering all the relevant congenic strains in the B6.C RQI b5i7 series, the mean congenic-vs.-background effect size for *D1Mit167* is $(\sum E_m^*)/n = 1.89$. The above individual and mean E_m* values are larger than the effect size obtained from marker class mean differences (E_m). The variation in effect size among the RQI congenic strains that carry a given significant QTL can be attributed to the relatively minor effect of the rest of the introgressed donor genome, which is about 3% in the b5i7 series on the average, to random environmental variation, experimental error in measuring the phenotype, etc. Thus, if only the detected QTL is carried by the congenic RQI strain, in the process of high resolution mapping, using a specific congenic, isogeneic RQI strain, we can expect and utilize a considerably larger effect size than the marker effect size detected in regression analysis with a population of RQI strains (E_m*>E_m). The effect of the frequency of recombination on QTL effect size was discussed in more detail by Lynch and Walsh (1998).

A large body of evidence implicates the mesolimbocortical dopaminergic system in alcohol-related behaviors (Koob et al., 1998). Recently, in an alcohol-preference study, we looked at the genetic (between-strain) correlation between alcohol preference and TH/MES, because the B6.C and B6.I RQI strains were developed by selection for the high and low expression of TH/MES, respectively. No significant correlation was detected in the B6.C RQI strains of the b4i5 series (Vadasz et al., 2000). In the present study with RQI strains of the b5i7 series, such correlation analysis could not be performed because no TH/MES data are available. However, we had the opportunity to compare the chromosome scans of the B6.C and B6.I strains, which differ in at least two aspects: (1) the donor strain and (2) the direction of selection for TH/MES (Fig. 3). Interestingly, no C-type loci could be detected on chrs. 2 and 9 in the B6.I set of strains, although the donor CXBI strain carries C-type genes and markers on both chromosomes. An intriguing possibility is that selection for low TH/MES eliminated the relevant C-type alleles, and the resulting difference in marker distribution may help in identifying chromosomes that carry QTLs for TH/MES. Other factors, however, may also be responsible for the difference. For example, unequal strain sample size (the number of strains is different in the B6.C and B6.I sets) and unequal frequency of C-type loci in the donor strains (CXBI, as an RI strain, is expected to carry 50% C and 50% B6 alleles).

Careful definition of the phenotype remains an important issue in the genetic analysis of alcohol-related behaviors. Ontogenesis, as pointed out by Kakihana and McClearn (1963), is a critical factor in the BALB/c strain, which was characterized with relatively high alcohol preference (0.5) at 4 weeks of age, and significantly lower preference (0.12) at

16 weeks of age, with an abrupt threshold at about 9 weeks of age. In our study, the 9–11-week-old BALB/c mice, as expected, showed low alcohol preference (Table 1). Functional genomic analysis of this very interesting developmental pattern in the relevant congenic B6.C RQI strains may help in clarifying its mechanism.

In conclusion, we identified provisional QTLs for EAC on chr. 1 close to the centromere of chr. 1 and near the telomere of chr. 2. These locations are consistent with previous reports (Rodriguez et al., 1995; Gill et al., 1998; Whatley et al., 1999). We suggest that these QTLs may correspond to kappa opioid receptor 1 and kappa opioid receptor 3, and may play a significant role in voluntary alcohol consumption. However, this hypothesis is not supported by direct physiological evidence because no RQI strains have been tested for differences in the relevant opioid characteristics. Also, among the genes located on the candidate segments [estimated as about 360 genes (on chr. 1, 8.25 cM) and 530 genes (on chr. 2, 12 cM), either other known genes (e.g., on chr. 1 Grip1, and Kcnb2, and on chr. 2 Gnas, Nztf2, and Acra4) or unknown genes might be implicated. Our provisional results need to be confirmed by further studies on segregating generations and other genetic preparations.

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