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Quantitative Trait Loci Associated with Short-term Intake of Sucrose, Saccharin and Quinine Solutions in Laboratory Mice

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Abstract

The goal of this study was simultaneously to map two genetic loci which, collectively, have a large effect on intake of sucrose, saccharin and quinine solutions in mice. These loci had been previously identified using long-term measurements with the traditional two-bottle test, but the present study used a short-term, one-bottle test. Intake of distilled water, 100 mM sucrose, 10 mM sodium saccharin and 1.1 mM quinine HCl over 6 h was measured on two occasions from a non-deprived group of 61 male and 72 female F_2 mice derived from a cross of the C57BL/6J and DBA/2J mouse strains and used to detect quantitative trait loci (QTL). DNA from each animal was typed for polymorphisms in anonymous microsatellite markers on mouse chromosomes 4 and 6. Saccharin and sucrose relevant QTL were detected on distal chromosome 4 and a quinine relevant QTL was detected on medial/distal chromosome 6 in the region of *Prp*. The location of these QTL and the proportion of phenotypic variance they accounted for were similar to those arrived at following previous determinations using the two-bottle test. Measurement stability for the three gustatory phenotypes was high, product-moment correlation coefficients between first and second determinations varying between ~0.80 for sucrose and saccharin and 0.73 for quinine. QTL parameters assessed independently for first and second presentations of sucrose and saccharin were stable, but the location of the quinine QTL differed between presentations. The present experiment illustrates the utility of a 6 h fluid intake test in the mapping of *Sac* and *Qui* loci. The short duration of the test provides a simple means of measuring variation in gustatory processes and the discovery that these loci influence short-term as well as long-term fluid intake extends understanding of the mechanism of gene action.

Introduction

Strain differences (Ninomiya and Funakoshi, 1993) and selection studies (Nachman, 1959; Dess and Minor, 1996) illustrate the ubiquitous contribution of genes to taste processes. However, although the underlying genetic variations constitute a pool of enormous potential, until recently, methodological limitations have rendered it impossible to explore the underlying genetic architecture—the number of genes, the magnitude of effect associated with allelic variation, and dominance relations at individual loci. Gene-mapping, or reverse genetics, has dramatically changed the ability of the experimenter to decompose these behavioral variations between strains to identify the contribution of individual genes to behavior (Blizard and Darvasi, 1999).

The aim of taste genetics is not merely to establish the existence of genes related to taste physiology and behavior, but to facilitate cloning and, ultimately, understanding of the cellular and molecular processes underlying taste-mediated processes. By linking taste genes to markers whose chromosomal location is known, the stage is set for fine-mapping and cloning of the taste genes themselves.

When two inbred strains are crossed, the F_1 progeny are all essentially identical and heterozygous at all genetic loci where the parents had different alleles. The F_2 generation, however, is genetically diverse as a result of random chromosomal assortment as well as chromosomal rearrangement of crossing over during duplication. Hence, the F_2 generation is suitable for studying genetic variability because the population has the same markers and genes as the parents but in different combinations. These combinations serve to establish the linkage between the taste-related genes and the known markers.

Traditional genetic studies that relied on behavioral measurements of F_2 generations had deficiencies in the measurement of both genotype and phenotype. The genotype often could not be established unequivocally in the absence of suitable markers; phenotype (a term used by

geneticists to emphasize that the visible properties of an organism reflect the interaction of genetic and environmental influences) usually was assessed only in a qualitative manner. Newer methods using polymerase chain reaction (PCR) amplification of DNA can directly establish the genotype of individual animals for which molecular biological markers are known. These may be known genes with established functions, or may be so-called 'microsatellite markers' which are distinctive repeating nucleotide combinations occurring at intervals throughout the genome. New methods also improve phenotypic assessment by analyzing traits in a quantitative manner and parsing the contributions of different genes (or chromosomal loci) with the quantitative measurement of phenotype. The quantitative trait locus (QTL) method (Lander and Botstein, 1989) employs statistical programs such as Mapmaker to establish the chromosomal locations of genes that make statistically significant contributions to the behavioral phenotype.

Recombinant-inbred (RI) strains (Bailey, 1971; Taylor, 1978) have been used frequently as genetic mapping tools in studies of taste. They consist of multiple inbred strains, each of which has been independently derived from a cross of two genetically diverse inbred strains. As a result of inbreeding, each RI strain becomes fixed, i.e. homozygotic, at all loci. After genotyping on numerous markers, sets or batteries of RI strains derived from the same pair of progenitor strains, constitute a fixed repository of genotypes which can be correlated with phenotypic measurements on those strains. RI strains therefore permit analysis of the relationship between genotype and physiological and behavioral characters without additional genotyping. For further discussion of the advantages of RI strains see Bailey (Bailey, 1971) and Taylor (Taylor, 1978).

Assuming single or major gene control and using qualitative descriptions of phenotypes (+, –), Azen *et al.* (Azen *et al.*, 1986), Lush and co-workers (Lush and Holland, 1988; Lush, 1991) and Capeless *et al.* (Capeless *et al.*, 1992) have implicated the *Prp* region on mouse chromosome 6 in intake of quinine, sucrose octa-acetate (SOA) and other aversive solutions. In addition, on the basis of characterizations of RI strains derived from C57BL/6J (B) and DBA/2J (D) strains and backcross mice, Lush *et al.* (Lush *et al.*, 1995) were able to identify distal chromosome 4 as the most likely location for the *Sac* locus (Fuller, 1974). Similarly, Ninomiya *et al.* used categorical assignment procedures to map a gene, *dpa*, on proximal chromosome 4, but used a different behavioral phenotype, i.e. variation in generalization of 0.1 M D-phenylalanine (D-phe) to 0.5 M sucrose (Ninomiya *et al.*, 1991).

The more flexible approach to linkage detection, QTL analysis (Lander and Botstein, 1989), has been used more sparingly in the field of gustatory research. In QTL analysis, an individual's phenotypic score is assumed to reflect the net influence of allelic variation in the individual components of a polygenic system which includes systematic and random environmental variations to the phenotype. QTL analysis is especially appropriate for complex traits, including behavioral and physiological processes, such as those involved in gustation, because these are usually assumed to be controlled by many genes. Again, the principal approach has been via RI strains (Plomin *et al.*, 1991) and has been applied to both 'sweet' (Belknap *et al.*, 1992; Phillips *et al.*, 1994) and 'bitter' (Harder and Whitney, 1998) solutions; a variety of QTL sites have been identified. The study by Phillips *et al.* (Phillips *et al.*, 1994) is one of the few QTL studies to use female mice and is particularly interesting for that reason. RI–QTL studies are particularly useful in nominating QTL sites for further study. However, both simulation (Belknap *et al.*, 1996) and empirical (Tarantino *et al.*, 1998) studies suggest that because of the large number of statistical comparisons involved and the small size of most RI sets, these nominations are susceptible to both false positive and false negative (Type I and Type II) errors.

QTL analysis also can be conducted through analysis of F_2 or backcross populations which can more flexibly address statistical power requirements, as well as permitting exploration of dominance relations between alleles. Two brief reports (Bachmanov *et al.*, 1996; Blizard *et al.*, 1996) introduced the QTL approach via F_2s to gustatory research, including a recent full length report (Bachmanov *et al.*, 1997). In the present experiment, previously presented in abstract (Blizard *et al.*, 1996), we introduce a novel behavioral phenotype—short-term intake from a single tube by non-deprived animals (Kotlus and Blizard, 1998)—to determine whether this faster test detected previously reported QTL on chromosomes 4 and 6. Also, we studied several gustatory phenotypes in order to evaluate the potential for detecting multiple QTLs in the same group of animals. Finally, the mapping population included both males and females to broaden the scope of generalization concerning QTL previously detected.

Materials and methods

Animals and maintenance procedures

Inbred mice from the B (two males, three females) and D (seven males, four females) strains, the B/D F_1 (nine males, nine females derived from a unidirectional cross) and B/D derived F_2 (61 males, 72 females) were subjects for this experiment. They were bred at the Center for Developmental and Health Genetics Animal Laboratory at Pennsylvania State University. A 12 h/12 h light/dark cycle was maintained in the animal room. Temperature was controlled at 70 ± 1°F. After weaning at 25–28 days, animals were individually housed in clear plastic cages on wood-chip bedding until 60 days of age, when fluid intake tests for alcohol preference and alcohol acceptance were administered; for a description of procedures see Rodriguez *et al.* (Rodriguez *et al.*, 1994). Forty days after completion of

these tests, when mice were ~130 days old, the taste tests described below were administered. One week before the beginning of the tests, the mice were switched from tap to distilled water and this regime was continued until the completion of the experiment.

Fluid intake test

A short-term, fluid intake test was used to characterize individual variation in intake of 'sweet' and 'bitter' tastants. In this paper 'sweet' and 'bitter' are used to refer to chemical solutions usually classified with those descriptors by humans. Use of these terms to describe solutions is not intended to imply that the human taste qualities of sweet and bitter have counterparts in animals.

The rationale for the test has recently been described (Kotlus and Blizard, 1998) and estimates of measurement stability provided. The test presents solutions to non-deprived animals in single, 25 ml, graduated tubes in the position on the home-cage normally occupied by the water-bottle. Intakes of relevant tastants are recorded for a 6 h period beginning 3 h prior to the onset of the 12 h dark phase, thus ensuring that data collection coincides with the phase in the circadian cycle when mice begin to drink. In the 18 h inter-test period animals are maintained on distilled water *ad libitum*. The solutions were given on consecutive days in the following order: distilled water (DW), sucrose (100 mM), sodium saccharin (10 mM), quinine HCl (1.1 mM), and then repeated, the complete sequence lasting 8 days. Mice drink approximately 1.0–2.0 ml of water, 1.0–10 ml of sucrose or saccharin and from 0 to 1.5 ml of quinine HCl in the 6 h period.

Genotyping

DNA was extracted from tail tips (Laird *et al.*, 1991). Anonymous microsatellite markers (Research Genetics, Huntsville, AL) were used to type DNA from each animal. Seven markers were used to type DNA on chromosome 4, and five on chromosome 6. Care was taken to include markers which were close to the sites of *Sac, Qui* and *dpa* (see QTL section of Results). Amplification was performed on a Perkin-Elmer 9600 thermocycler. The total volume of the reaction mixture was 12.5 ml, consisting of 30 ng genomic DNA, 0.4 μm primers, 0.2 mM of each dNTP, 0.5 U Taq polymerase (Perkin-Elmer Cetus, Foster City, CA), 10 mM Tris–HCl, pH. 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.16 mg/ml bovine serum albumen (BSA). Thermal cycling included a 5 min. denaturation step at 94°C, 35 cycles of 30 s at 94°C, 30 s at 53°C, 30 s at 72°C and a final extension step for 10 min. at 72°C. PCR products were separated on 4% agarose gels and visualized with ethidium bromide staining. Inbred strain (B and D) and heterozygote DNA were included as standards with each batch of experimental samples. The gels were photographed to provide a durable record of the results.

Statistical analysis

Statistical analysis of intake data

Statistical analyses were conducted with the Systat for Windows software package (Wilkinson *et al.*, 1992). F ratios and the degrees of freedom for numerator and denominator derived from analyses of variance are presented in the following manner: [$F(1,130) = 26.27$].

QTL analysis

Linkage between markers was determined using the IBM-compatible Mapmaker/EXP (version 3.0) software package using a maximum likelihood procedure. QTL analyses of the solution intakes were performed using Mapmaker/QTL (version 1.1), which employs an interval mapping method to determine the position of a QTL and the proportion of phenotypic variance that may be attributed to it (Lander *et al.*, 1987). The principal parameter used by Mapmaker to determine statistical significance is the LOD score (log of the odds ratio). This expresses the likelihood of the observed data (assuming the presence of a QTL at a particular location) divided by the likelihood of no QTL at that position (the null hypothesis) as log to the base 10. Thus, a LOD score of 3.0 represents an odds ratio of 1000 to 1 that a specific QTL exists at a given location. Due to the multiple comparisons which occur during whole genome scans a 'protected' LOD score of 4.3 has been suggested (Lander and Kruglyak, 1995) to equate to the ($P < 0.05$) conventional two-tailed level of significance in an F_2 intercross (where there are three independent genetic classes and therefore $n - 1$, or 2 degrees of freedom). Mapmaker conducts separate analyses according to different assumptions about dominance relations at each marker: free, degree of dominance estimated from the data; dominant, B allele completely dominant; recessive, B allele completely recessive; additive, heterozygote is the mean of homozygotes. For one model to be preferred over another, convention requires a difference between LOD scores ≥1.0. The 1-LOD support interval (which approximates the 95% confidence interval) is estimated as the interval defined by values on the QTL plot at 1 LOD below the peak LOD score.

Results

Inbred strains and F_1s

Repeated measures analysis of variance of first and second presentations of the four solutions only found a statistically significant change in the case of sucrose. For this solution, intake on the second presentation was significantly higher [$F(1,28) = 6.62$, $P < 0.02$]. There was no interaction of day of presentation with strain or gender. Pearson product-moment correlations (r) of the pooled data revealed high positive correlations between first and second presentations of all three flavored solutions (sucrose, 0.88; saccharin, 0.85; quinine, 0.73; $P < 0.0001$ in all cases), but a low correlation (0.28, $P < 0.12$) between first and second water intakes.

Table 1 Intercorrelation between combined water, sucrose, saccharin and quinine intakes in C57BL/6J, DBA/2J and B6/D2 F_1s

Solution	Water	Sucrose	Saccharin	Quinine
Water	1.000			
Sucrose	0.237	1.000		
Saccharin	0.294	0.942***	1.000	
Quinine	0.659***	0.120	0.190	1.000

***$P < 0.0001$.

Table 2 Mean of combined intakes (ml/mouse/12 h) ± SE of four solutions in two inbred strains and their F_1 hybrid

	C57BL/6J ($n = 5$)	B6/D2 F_1 ($n = 18$)	DBA/2J ($n = 11$)
Water	2.32 ± 0.20	2.81 ± 0.18	2.75 ± 0.12
Sucrose	9.78 ± 1.33	10.31 ± 0.88	3.20 ± 0.31
Saccharin	8.76 ± 0.80	8.98 ± 0.66	3.30 ± 0.29
Quinine	0.56 ± 0.17	2.13 ± 0.23	1.84 ± 0.24

Intakes for first and second presentations of each solution were therefore summed for statistical analysis. Pearson product-moment correlational analysis of the combined scores revealed a high positive correlation between sucrose and saccharin intake and between water and quinine intake (Table 1). These high correlations are likely the result of associations among strain means in the different measures of intake rather than individual variability within genotypes.

The combined intakes of the three different flavored solutions are shown in Table 2. There were no statistically significant differences between males and females in intake of any of the solutions, so gender was ignored in subsequent analyses. Analysis of variance revealed highly significant differences between the three genetic groups [$F(2,31)$ (sucrose = 20.15, $P < 0.0001$; saccharin = 23.43, $P < 0.0001$; quinine = 6.47, $P < 0.004$)]. *Post-hoc* comparisons using the Bonferroni protection procedure showed that B mice drank more sucrose ($P < 0.001$) and saccharin ($P < 0.0001$) and less quinine ($P < 0.03$) than D mice. F_1 intake of the 'sweet' solutions did not differ significantly from that of B mice and was higher than that of D mice (sucrose and saccharin, $P < 0.0001$). F_1 intake of quinine was similar to the level exhibited by D mice, differing significantly from B mice ($P < 0.003$). There were no statistically significant differences in 6 h water intake between the groups ($P < 0.31$). Separate analyses of variance of first and second presentations of each solution yielded similar group differences to those provided by analyses of combined intakes ($P < 0.0001$ for both presentations of sucrose and saccharin; $P < 0.002$, $P < 0.04$ for first and second presentations of quinine; water, not significant on both occasions).

Considering changes from baseline water intake, Table 2 shows clearly that B and F1 mice drank more of both sucrose and saccharin solutions. Repeated measures analyses of variance of intakes within the D strain suggested that the D mice ($n = 11$) also drank more of the 'sweet' solutions than water [$F(1,10)$ (sucrose, = 4.01, $P < 0.07$; saccharin = 5.87, $P < 0.04$)] but that the magnitude of the increase was modest and statistical significance was borderline. Table 2 also shows that B mice drank less quinine than water. Repeated measures analysis of combined quinine intakes indicated that D and F_1 mice also drank significantly less of the quinine solution than water [D mice, $F(1,10) = 30.01$, $P < 0.0001$; F_1 mice, $F(1,17) = 16.16$, $P < 0.001$)], these levels of significance obviating the need for *post hoc* protection.

F_2 generation

Repeated measures analyses of variance of first and second presentations of the four solutions found a statistically significant change for all three solutions: sucrose [$F(1,127) = 14.02$, $P < 0.0001$], saccharin [$F(1,127) = 18.9$, $P < 0.0001$], quinine [$F(1,126) = 3.92$, $P < 0.05$] and water [$F(1,131) = 51.13$, $P < 0.0001$]. Sucrose (3.62 versus 4.15 ml), saccharin (3.79 versus 4.34 ml) and water (1.10 versus 1.40 ml) intakes increased on the second presentation while quinine intake (0.78 versus 0.73 ml) decreased. There was no interaction of presentation order with gender or genotype at the microsatellite marker that was later found to best predict intake of the different solutions (see below). In spite of these changes in intake which occurred between presentations, correlational analysis revealed similar relationships between first and second presentations of the three solutions to that found among the inbred strains and F_1s [r(sucrose, 0.79; saccharin, 0.81; quinine, 0.73; $P < 0.0001$)] in all cases. First and second water intakes were also significantly correlated ($r = 0.45$, $P < 0.0001$) at a slightly higher level than that seen in analyses of the fixed genotypes.

Intakes for first and second presentations of each solution were therefore summed for statistical analysis. Analysis of the combined intakes (Table 3) shows a strong positive relationship between sucrose and saccharin intakes, a modest positive correlation between either sucrose or saccharin intake and quinine intake; and water intake correlated modestly with intake of all three flavored solutions. To explore whether the relationship of quinine to sucrose and saccharin intake was dependent on their common relationship to water intake, the three variables were regressed on water intake and the relationships among the residuals explored. A small positive relationship was preserved among residuals [r(sucrose/quinine = 0.21, $P < 0.022$; saccharin/quinine = 0.26, $P < 0.005$)].

The covariation among fluid intakes was not based on variation in body weight which did not correlate significantly with intake of any of the solutions when data from the

Table 3 Intercorrelation between combined water, sucrose, saccharin and quinine intakes in 133 B/D F_2s

Solution	Water	Sucrose	Saccharin	Quinine
Water	1.00			
Sucrose	0.34***	1.00		
Saccharin	0.34***	0.91***	1.00	
Quinine	0.33***	0.30**	0.34***	1.00

***$P < 0.0001$; **$P < 0.001$.

entire group were analyzed. Among males, there was no significant relationship between body weight and any measure of fluid intake, and significant correlations with body weight among females were restricted to combined sucrose ($r = 0.34$, $P < 0.004$) and combined saccharin intakes ($r = 0.39$, $P < 0.001$).

Analyses of variance, carried out to assess the contribution of gender to each variable, revealed that female mice exhibited slightly higher fluid intakes than males [$F(1,131)$ (water = 3.93, $P < 0.05$, male 2.36 ± 0.09, female 2.63 ± 0.09; sucrose = 4.05, $P < 0.05$, male 6.98 ± 0.54, female 8.44 ± 0.49; saccharin = 6.34, $P < 0.01$, male 7.19 ± 0.51, female 8.93 ± 0.47)]. There was no gender difference in intake of the quinine solution [$F(1,131) = 1.62$, $P < 0.21$].

As noted above, water intake was associated significantly with intake of all three solutions in the F_2 generation. To assess the appropriateness of adjusting intake of the three solutions for baseline water intake in QTL analyses, we calculated product-moment correlation coefficients between water and solution intake within the three allelic classifications (BB, BD, DD) for those markers which were later found to be the best predictors of intake of the 'sweet' and 'bitter' solutions (*D4Mit42* for sucrose and saccharin, *D6Mit338* for quinine). The numbers of animals within these genotypic classes are recorded in Table 6. For the *D4Mit42* marker, correlations were statistically significant among mice which were DD homozygotes [r(water/sucrose = 0.61, $P < 0.0001$; water/saccharin = 0.59 $P < 0.0001$)], but not statistically significant or of borderline significance among mice which were heterozygotes [r(water/sucrose = 0.25, not significant; water/saccharin = 0.27, not significant)] or BB homozygotes [r(water/sucrose = 0.26, not significant, water/saccharin = 0.31 $P < 0.05$)]. For the *D6Mit338* marker there was a statistically significant relationship between intake of water and quinine among DD mice [r(water/quinine = 0.35, $P < 0.05$)] and heterozygotes: [r(water/quinine = 0.38, $P < 0.002$)], but not among BB homozygotes [r(water/quinine = 0.27, not significant)]. In all of these cases, possession of the allelic variant which was associated with a large deviation of solution intake from baseline water intake perturbed the 'usual' relationship between intake of water and flavored solutions.

The effect of gender on solution intake was also dependent on genotypic class. For both sucrose and saccharin solutions, females drank significantly more than males if their genotypic classification at *D4Mit42* was BB [$F(1,40)$ saccharin = 6.67, $P < 0.01$, male 8.03 ± 1.0, female 11.27 ± 0.75; sucrose = 4.04, $P < 0.05$, male 7.73 ± 1.09, female 10.46 ± 0.81)], exhibited a non-significant trend in the same direction if their genotype was BD (saccharin, male 8.08 ± 0.64, female 9.31 ± 0.0.70; sucrose, male 7.67 ± 0.69, female 9.09 ± 0.76), but did not differ from males if their *D4Mit42* classification was DD (saccharin, male 4.74 ± 0.66, female 5.29 ± 0.59; sucrose, male 4.96 ± 0.82, female 4.91 ± 0.74).

The variability in the relationship between water intake and the three other solutions within the different genotypic classes precluded use of water intake for the purpose of statistical adjustment. Gender corrections could not be applied for the same reason.

QTL analyses

Sucrose and saccharin intakes

Combined intakes were used for the primary Mapmaker analyses. Log transformations were also applied to intakes to remove slight deviations from normality.

As shown in Figures 1A and B, both sucrose and saccharin intakes were strongly related to variation on distal chromosome 4 (for sucrose intake the peak LOD score was 8.85 and for saccharin, 10.61). Sucrose and saccharin QTL accounted, respectively, for 27.7 and 31.2% of the phenotypic variance. Complete dominance of the B allele for increased intake was the model which provided the best fit for sucrose intakes. The peak LOD score of 8.45 for the dominant model did not differ significantly from the 'free' LOD score and exceeded that calculated for the additive model (7.41) by more than 1 log unit. The dominant model also provided the best fit for the saccharin data. Again, the relevant LOD score of 9.93 did not differ significantly from that calculated for the free model, but in this case did not differ significantly from that based on an additive model (LOD = 9.34). For both solutions, the recessive model was unambiguously rejected (see Figures 1 and 2). The statistical properties of the QTL on distal chromosome 4 are summarized in Table 4. The QTL peaks for both solutions were within 1 cM of *D4Mit42* at 81 cM which is close to the location estimated for the *Sac* locus (Lush *et al.*, 1995), but because no markers were typed distal to *D4Mit42*, the 1-LOD support interval or 95% confidence interval for the sucrose and saccharin QTL, represented in Figures 1 and 2 by the dark horizontal bar, only extends in a proximal or centromeric direction. A more realistic confidence interval for the sucrose QTL would likely project from *D4Mit33* + 2.5 cM to the end of the chromosome which is currently estimated by the Mammalian Genome Database (http://www.informatics.jax.org), to be 3.0 cM distal to *D4Mit42,* for a total of 10.4 cM. The analogous estimate for the sac-

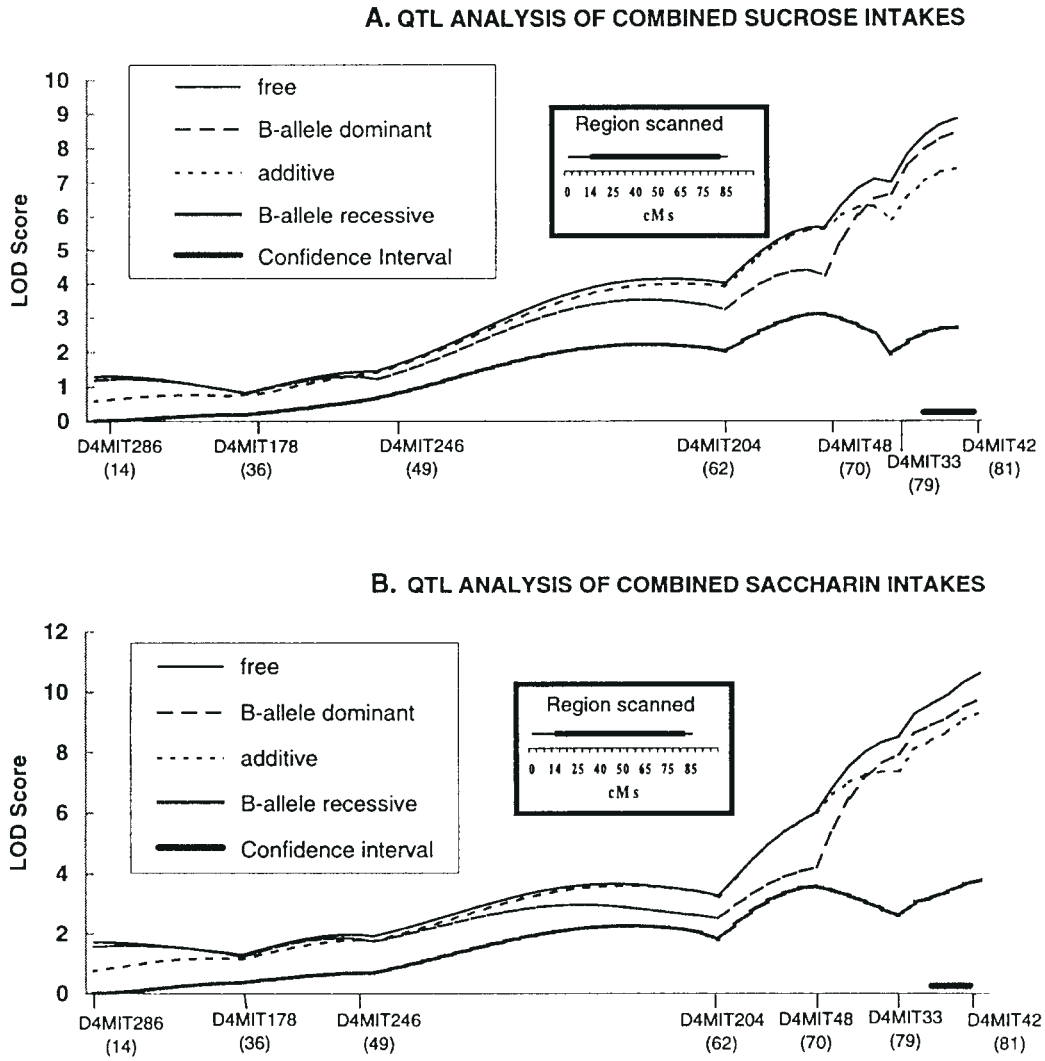

Figure 1 *x* axis: maximum likelihood linkage maps in centimorgans (cM) based on recombination frequencies estimated from the present data using Mapmaker EXP. The maximum likelihood gene order and percentage recombination between chromosome 4 markers estimated in the present study were (centromere to telomere) *D4Mit286*-18.4-*D4Mit178*-17.3-*D4Mit246*-43.3-*D4Mit204*-13.7-*D4Mit48*-10-*D4Mit33*-9.9-*D4Mit42*. The insert in the figures illustrates by a thickened line the region of chromosome 4 represented on the *x* axis. *y* axis: curves showing LOD scores (see text) for QTL affecting **(A)** 6 h intake of 100 mM sucrose and **(B)** 10 mM sodium saccharin in 133 (B × D) F_2 progeny. The four curves in each figure represent different assumptions regarding the dominance of the QTL allele inherited from B (see text). Numbers in parentheses on the *x* axis indicate the Mouse Chromosome Committee distances in cM from the centromere for each marker (http://www.informatics.jax.org). The thick horizontal lines on the right-hand side above the *x* axis indicate the 1-LOD support interval (95% confidence range) for the QTL.

charin QTL extends from *D4Mit33* + 4.0 cM to the end of the chromosome, a total distance of 9.4 cM.

No other sucrose or saccharin relevant QTL emerged from Mapmaker analyses of the other regions of chromosomes 4 and 6, including the region of chromosome 4 swept by *D4Mit286* at 14.5 cM which is ~4.0 cM proximal to the location for *dpa* suggested by Ninomiya *et al.* (Ninomiya *et al.*, 1991). However, given that the large effect of the QTL on distal chromosome 4 would appear in the error term of other QTL, sucrose and saccharin intakes were regressed on allelic status at *D4Mit42* (the marker closest to the relevant QTL peaks) and Mapmaker analyses conducted on the residuals. No additional peaks were detected on either chromosome.

Quinine intake

As shown in Figure 2, analysis of log-transformed, combined quinine intakes found a QTL in the region of *D6Mit338* on chromosome 6 with a peak LOD score of 4.73 which accounted for 19.3% of the phenotypic variance. *D6Mit338* at 62.3 cM is close to *Prp* at 63.6 cM. Additive (LOD = 4.56) and recessive models (LOD = 3.91) for the B allele for increased quinine consumption did not differ from the free model but the dominant model was rejected

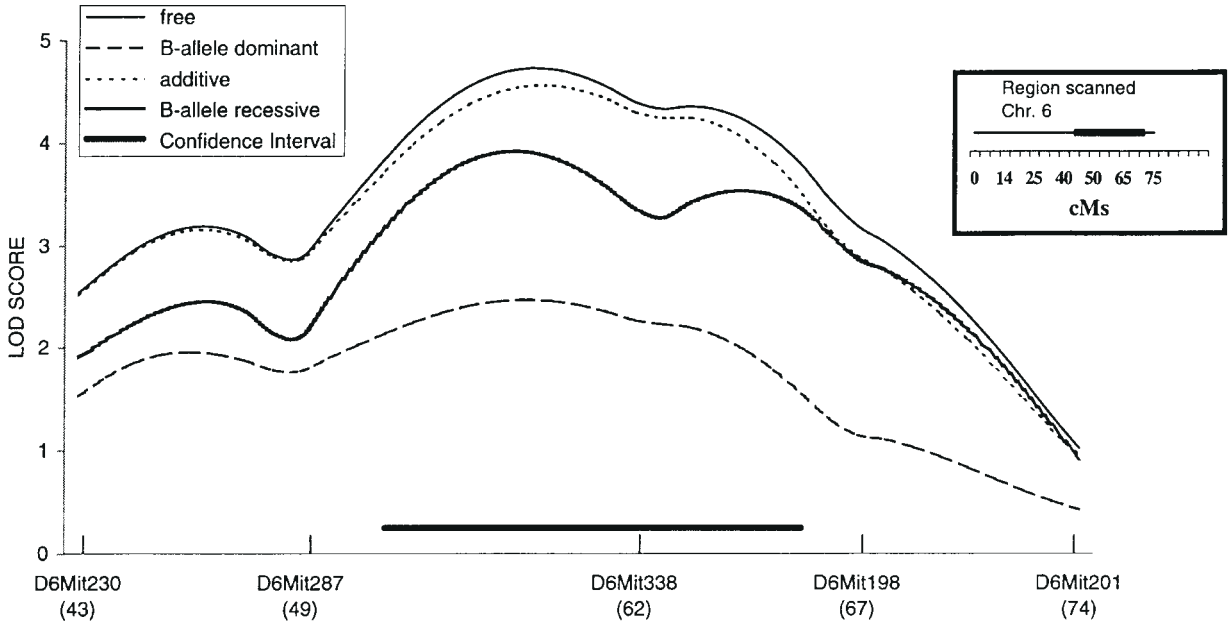

Figure 2 *x* axis: maximum likelihood linkage maps in centimorgans (cM) based on recombination frequencies estimated from the present data using Mapmaker EXP. The maximum likelihood gene order and percentage recombination between chromosome 6 markers estimated in the present study were *D6Mit230*-14.2-*D6Mit287*-24.8-*D6Mit338*-13.2-*D6Mit198*-16.1-*D6Mit201*. The insert in the figure illustrates by a thickened line the region of chromosome 6 represented on the *x* axis. *y* axis: curves showing LOD scores (see text) for QTL affecting 1.1 mM quinine HCl intake in 133 (B × D) F_2 progeny. The four curves represent different assumptions regarding the dominance of the QTL allele inherited from B (see text). Numbers in parentheses on the *x* axis indicate the Mouse Chromosome Committee distances in cM from the centromere for each marker (http://www.informatics.jax.org). The thick horizontal line above the *x* axis indicates the 1-LOD support interval (95% confidence range) for the QTL.

Table 4 Summary of QTL parameters for combined intakes of sucrose, saccharin and quinine

	LOD score (free model)	Variance explained (%)	QTL position in cM relative to Mit marker	Allelic model for increased consumption	LOD score for preferred model	1-LOD support interval (cM)
Sucrose	8.68	27.7	D4Mit33 + 9.0	B dominant	8.45	7.4[a]
Saccharin	10.75	31.2	D4Mit42	B dominant	9.93[b]	6.0[a]
Quinine	4.73	19.3	D6Mit287+ 16.5	Additive	4.56[c]	28

[a]The 1-LOD support interval within the *D4Mit33-D4Mit42* interval (see Figures 1 and 2). Because no markers were typed distal to *D4Mit42*, the portion of the 1-LOD support interval distal to this marker could not be estimated but probably extends to the end of chromosome 4, a distance of ~3.0 cM.

[b]As noted in the text, the LOD score for the dominant model for saccharin consumption did not differ by the 1-LOD criterion from the additive model.

[c]The LOD score for the additive model did not differ by the 1-LOD criterion from the recessive model.

(Table 4). The 95% confidence interval is represented by the dark horizontal bar.

No other QTL emerged from Mapmaker analyses of combined quinine intakes for other regions of chromosomes 4 and 6. In addition, when quinine intakes were regressed on their relationship to *D6Mit338* and the residuals subjected to Mapmaker analysis, no statistically significant LOD scores were detected. The only indication of the presence of QTL in analyses of adjusted data were LOD scores of 1.89 and 1.78, found, respectively, near *D4Mit246* and *D4Mit286* (although not at *D4Mit178* which is located between them). Although not statistically significant or even 'suggestive' using Lander and Kruglyak's criterion (Lander and Kruglyak, 1995), these findings are reported in case similar results are obtained by other investigators in the future.

QTL-stability

To provide information on QTL stability, Mapmaker analyses were conducted separately on intakes obtained during first and second presentations of the three solutions. As shown in Table 5, these revealed good agreement between QTL parameters (LOD score, proportion of phenotypic

Table 5 Summary of QTL parameters for first and second presentations of three solutions

	100 mM sucrose		10 mM saccharin		1.1 mM quinine	
	1st	2nd	1st	2nd	1st	2nd
LOD score	8.07	6.76	8.55	8.97	3.66	3.76
% variance explained	25.6	21.6	26.7	27.8	16.3	13.6
QTL-peak location	D4Mit33 + 8.8	D4Mit33 + 8.2	D4Mit33 + 8.1	D4Mit33 + 9.6	D6Mit287 + 14.3	D6Mit338 + 6.4

Table 6 Mean of combined intakes (ml/mouse/12 h) ± SE of three solutions in F_2 mice classified by genotype at the *D4Mit42* (sucrose and saccharin) and *D6Mit338* (quinine) microsatellite markers

D4Mit42 status (*n*)[a]	BB (42)	BD (55)	DD (36)
Sucrose	9.48 ± 0.60***	8.32 ± 0.52***	4.93 ± 0.64
Saccharin	10.11 ± 0.55***	8.64 ± 0.48***	5.05 ± 0.59
D6Mit338 status (*n*)	BB (30)	BD (69)	DD (33)
Quinine	1.04 ± 0.15	1.52 ± 0.10*	1.91 ± 0.14***

*$P < 0.05$, ***$P < 0.0001$ versus BB by Bonferroni *post hoc* test.

[a]The frequencies of the three genotype classes for this marker did not differ significantly from Mendelian ratios by χ^2 test, $P < 0.09$.

variance accounted for, and QTL location) for first and second presentations of sucrose and saccharin.

Also shown in Table 5 are the QTL parameters for quinine on tests 1 and 2. In this case, the LOD score and proportion of phenotypic variance accounted for were similar on both test occasions, but the QTL peak in test 1 was to the left of *D6Mit338* and to the right of the same marker in test 2.

Analyses of variance of F_2 data

Table 6 shows the mean differences in solution intake associated with allelic variation at markers closest to the QTL peaks. Log transformations of the raw data removed slight deviations from normality and were uniformly adopted before proceeding with statistical analyses. Analysis of variance of intake of 'sweet' solutions using allelic variation at the *D4Mit42* locus as the independent variable yielded highly significant F ratios [$F(2,130)$ sucrose = 23.46, $P < 0.00001$; saccharin = 29.37, $P < 0.00001$]. Bonferroni-protected *post hoc* tests indicated that both BB and BD mice (at *D4Mit42*) drank more of both solutions than DD mice ($P < 0.0001$ in all comparisons). Analysis of variance of quinine intakes, using allelic status at the *D6Mit338* locus as the independent variable also yielded a significant difference between the groups [$F(2,130) = 10.52$, $P < 0.0001$]. Bonferroni-protected *post hoc* tests indicated that BB mice (at *D6Mit338*) drank less quinine than BD ($P < 0.006$) and DD mice ($P < 0.0001$), while the difference between BD and DD groups did not reach statistical significance ($P < 0.09$).

Paralleling the Mapmaker analyses of the adjusted quinine intakes where a non-significant trend had been noted on chromosome 4, analysis of covariance of quinine intake using *D6Mit338* as the covariate gave the following results: *D4Mit246*, $F(2,128) = 2.70$, $P < 0.07$; *D4Mit286*, $F(2,128) = 2.24$, $P < 0.11$. The means and standard errors in quinine intake associated with these differences were as follows: *D4Mit246* (BB 1.25 ± 0.15; BD 1.55 ± 0.10; DD 1.63 ± 0.13); *D4Mit286* (BB 1.21 ± 0.16; BD 1.59 ± 0.10; DD 1.57 ± 0.12).

Summary

In summary, among male and female offspring of an intercross of B and D mice, ~30% of the total phenotypic variation in sucrose and saccharin intake during a 6 h test period appears to be determined by the same locus or loci on distal chromosome 4. Analogously, for quinine intake, a locus or loci on medial/distal chromosome 6 accounts for ~20% of the variation. The bulk of the evidence supported a dominant role for the B allele on chromosome 4 in increased intake of sucrose and saccharin, while intermediate or additive inheritance (defined as the midpoint between the B and D alleles) of the alleles on chromosome 6 provided the best model to account for variation in quinine intake.

Discussion

Short-term test of fluid intake

Previous research using long-term two-bottle preference procedures has implicated specific regions of mouse chromosomes 4 and 6, respectively, in intake of 'sweet' (Belknap *et al.*, 1992; Phillips *et al.*, 1994; Lush *et al.*, 1995; Bachmanov *et al.*, 1997) and 'bitter' (Azen *et al.*, 1986; Lush and Holland, 1988; Lush, 1991; Capeless *et al.*, 1992; Harder and Whitney, 1998) solutions. We mapped QTL which influenced 6 h intake of sucrose, saccharin and quinine solutions to the same chromosomal regions. Thus, both long- and short-term tests are influenced by at least two of the same genetic variations.

Fuller (Fuller, 1974) reported complete dominance of the B allele for increased saccharin consumption based on his comparisons of the B and D parental strains and their F_1 hybrid. More specifically, Bachmanov *et al.* (Bachmanov *et al.*, 1997) reported that the same dominance model fitted allelic interactions at the *Sac* locus on distal chromosome 4 in their study of two-bottle sucrose consumption in F_2 mice derived from a cross of C57BL/6By and 129/J strains. Using the B and D strains, we found allelic variation on distal chromosome 4 governing 6 h intake of both sucrose and saccharin to be best described by the same dominant model, although an additive model for saccharin consumption could not be rejected. For quinine consumption, the present experiment found that an additive model best described allelic influences at the QTL on chromosome 6, although a recessive mode of action for the B allele for increased quinine consumption could not be rejected.

The proportion of phenotypic variance accounted for by and the location of the *Sac* locus were similar to previous estimates by Bachmanov *et al.* (Bachmanov *et al.*, 1997). Thus, inclusion of both males and females in the present experiment was not associated with a disproportionate loss of statistical power. This would suggest that any variation associated with the estrous cycle or any other sexually dimorphic character did not substantially increase the phenotypic variance. Inclusion of both genders in future studies would increase knowledge of the role of gender in genetically based individual variation in gustatory processes.

The short-term test provides more specific information regarding the time required for the genes to manifest their effects. By comparison, the two-bottle procedure provides information regarding the stability of the variation in fluid intake over substantial periods of time. Thus, the results obtained with the different measurement procedures are usefully supplementing each other. Fluid intake during the 6 h test occurs mainly during the 3 h dark phase after 'light-off' (D.A. Blizard, unpublished results). Nevertheless, both 3 and 6 h periods are long enough to permit fluid intake to be influenced by post-ingestional factors (Davis, 1973). These could include the physiological effects of the particular solution ingested (Beauchamp and Fisher, 1993), or the effects of food-intake on fluid consumption (Kotlus and Blizard, 1998). Despite these limitations, the 6 h test provides a reliable short-term screen of fluid intake from a large number of animals using a discrete trial approach and is eminently suitable for gene-mapping experiments of the type described in this report.

Multiple phenotyping

The present experiment used a multiple phenotyping strategy encompassing solutions with contrasting effects on behavior in an effort to increase the return from the large effort involved in genotyping. The results affirm its utility: QTL predicted to occupy specific chromosomal regions were unambiguously detected and they accounted for approximately the same degree of phenotypic variance estimated in previous determinations. In addition, although analyses of variance found statistically significant differences between first and second presentations of the three flavored solutions, the QTL for sucrose and saccharin were reliably detected on each of the two measurement occasions. In the case of quinine intake, LOD scores did not reach acceptable levels of statistical significance on both measurement occasions and the estimated location of the QTL shifted. Without additional data, it is impossible to determine if the quinine results are due to variation introduced by the particular sequence of solutions used or simply from the loss of reliability resulting from use of one versus combined samples of fluid intake.

In our analyses of intakes combined across both presentations, we discovered that taste genes can be mapped reliably even when contrast effects, other sequence effects, and access to ethanol 1 month before could have contributed to total phenotypic variability. This means that the relevant genes influence intake of 'sweet' and 'bitter' solutions in a variety of circumstances. Nevertheless, multiple phenotyping may not always prove to be a good experimental strategy. Although the combination of solutions used in this experiment and the specific sequence that was used did not have a major impact on QTL detection, different combinations and sequences may.

Multiple phenotyping also had the additional benefit of revealing a statistically significant positive correlation in the F_2 group between the two 'sweet' solutions, on the one hand, and quinine, on the other, even after adjustments for their relationship to water intake. This is a phenotypic correlation and therefore could be caused by either genetic or environmental influences. The differences in intake between first and second presentations of all solutions in F_2 mice bespeak an environmental influence (for example, contrast or sequence effects) which could contribute to the association. Alternatively, the putative influence of genes such as α-gustducin on intake of both 'sweet' and 'bitter' solutions provides a genetic basis for such an association (Wong *et al.*, 1996).

Mechanisms of gene action at the *Sac* locus

Frank and Blizard (Frank and Blizard, 1996, 1999) showed that chorda tympani (CT) response to sucrose and saccharin was greater in B than D mice and that the CT threshold to sucrose was also lower in B mice. In addition, Bachmanov *et al.* (Bachmanov *et al.*, 1997) showed that the C57BL/6By strain (which is closely related to the B strain used in our experiment) exhibited similar differences in CT response to sucrose compared to the 129/J strain. Both experiments draw attention to the periphery as a possible site of action of the *Sac* locus. However, strain comparisons between inbred strains of diverse genetic origin necessarily involve confounding of differences at a specific genetic locus with many other allelic differences. Unless there is only one gene

in a cross that contributes to the relevant phenotypic variation, strain comparisons are only the first step in identifying biological mechanisms for further study. In the Bachmanov *et al.* (Bachmanov *et al.*, 1997) study, it was estimated that the two QTL which influenced intake of sucrose accounted for some 50% of the genetic variance. The inference to be drawn is that there are additional genes that account for the residual genetic variance in sucrose intake. In efforts to identify these, two RI–QTL studies (Belknap *et al.*, 1992; Phillips *et al.*, 1994) have nominated possible sites (aside from chromosome 4) on chromosomes 1, 3, 6, 8, 9, 12, 13, 15 and 18 governing two-bottle saccharin consumption in the B/D lineage.

Thus, strain comparisons of possible mechanisms need to be supplemented by studies in which allelic variation at a specific locus are used as the independent variables. A pioneering example of this is the experiment by Bachmanov *et al.* (Bachmanov *et al.*, 1997) who studied CT response in F_2 animals derived from an intercross of C57BL6/By and 129/J strains and found that the number of B alleles at the *D4Mit42* marker on distal chromosome 4 was positively correlated with the relative magnitude of CT response to supra-threshold sucrose solutions. This evidence supports the working hypothesis that variants at the *Sac* locus exert their effect by altering the peripheral receptive apparatus in the two strains. It seems highly likely that a similar relationship between allelic variants at the *Sac* locus and CT response exists in the B and D strains. The B/D strain difference in CT response mirrors that found between C57BL/6By and 129/J strains. In addition, careful comparisons by Capeless and Whitney (Capeless and Whitney, 1996) have documented close parallels between the two-bottle saccharin intake phenotype exhibited by D and 129/J strains.

That the *Sac* locus exerts its influence upon one or more peripheral gustatory processes is consistent with the fact that two of the three solutions (saccharin, sucrose and acesulfame) whose intake has been shown to be influenced by *Sac* (Lush *et al.*, 1995; Bachmanov *et al.* 1996, 1997; Frank and Blizard, 1996, 1999) are artificial sweeteners. Hence, it is highly unlikely that the alterations in intake associated with *Sac* are related to metabolic processes or post-ingestional effects. The 'peripheral' interpretation is also supported by the results of the present experiment in which a short-term test designed to minimize post-ingestional influences was used and *Sac* exerted the same magnitude of effect previously seen in studies which measured intake over periods of days.

Mechanisms of gene-action at the *Qui* locus

Frank and Blizard (Frank and Blizard, 1996, 1999) reported that, relative to B mice, D mice exhibit lower aversion and a greater CT response to quinine. Thus, assuming a simple relationship between the degree of quinine aversion and activity in the chorda tympani, a peripheral site of action for *Qui* does not seem to be a reasonable working hypothesis. However, there may be a variety of genes in the B/D lineage that exert effects on quinine intake and interpretation of the relationship between *Qui* and CT response to quinine must await their investigation. No whole genome scan of quinine intake in the B/D lineage has been published. However, a recent RI–QTL study of quinine and propylthiouracil (PROP) intake in the BXH RI set by Harder and Whitney (Harder and Whitney, 1998) nominated locations on chromosomes 3, 6, 7, 8 and 9.

The region of chromosome 6 defined by the *Qui* confidence interval has also been associated with variation in intake of other 'bitter' solutions, including cycloheximide, raffinose acetate and glycine in the B×D lineage (Lush, 1986; Lush and Holland, 1988), and with SOA and PROP in other lineages (Capeless *et al.*, 1992; Harder and Whitney, 1998). Lush (Lush, 1984, 1986) has speculated that the results obtained within the B×D lineage are consistent with the presence of a family of closely linked genes, each with varying degrees of specificity for the different solutions, a view which has been sharply challenged by Harder and Whitney (Harder and Whitney, 1998) who questioned the independence of these loci and offered a more parsimonious view in which the associations of this region of chromosome 6 with variation in intake of 'bitter' solutions was more plausibly understood as the result of allelic variation at a single locus, *Soa*. The effects of *Soa* are proposed to reflect allelic variation in the closely linked *Prp* family of genes, which control the secretion of proline-rich proteins in saliva (Azen *et al.*, 1986; Azen, 1991). Consistent with this, haplotype variation in *Prp* across several mouse strains predicts SOA aversion to a remarkable degree (Harder *et al.*, 1992). Nevertheless, direct evidence of a functional link between SOA aversion and the *Prp* gene has not yet been obtained.

Soa and *Qui* map to the same location of mouse chromosome 6 (albeit in different lineages) and may be the same gene (Harder and Whitney, 1998). However, until identity is established it seems useful to maintain separate nomenclatures. A compelling reason is that compared to D mice, B mice strongly avoid quinine while the opposite relation of the two strains exists regarding two-bottle intake of a 1 mM sucrose octa-acetate solution (Harder *et al.*, 1992). If the same gene accounts for the contrasting strain variations, its mechanism of action will need to be understood in enough detail to explain its opposite effects on intake of two 'bitter' solutions.

To summarize, although there are several promising avenues of investigation for both *Sac* and *Qui*, it is not yet known how the allelic variants exert their effect at either locus. Among other possibilities, anatomic differences in the number of taste buds, receptors or modality-specific primary afferents could alter, separately or collectively, the magnitude of the neural response to supra-threshold solutions and be a plausible peripheral site for gene action.

Alternatively, in the absence of anatomical differences, the allelic variants at *Sac and Qui* loci could alter the biochemical events (for example, Spielman *et al.*, 1994) which link receptor occupation to stimulation of an action potential in the afferent neuron.

Sac and *Qui*: a human perspective

It is conceivable that *Sac* and *Qui* have homologues in other species, including humans. If so, mutations at these loci may contribute significantly to the individuality of human gustatory sensibility. As discussed by Bachmanov *et al.* (Bachmanov *et al.*, 1997), knowledge of the chromosomal locations of genes in mice is helpful in suggesting regions of the human genome that may contain homologues of the relevant genes. A logical extension of the QTL approach is to fine-map the present loci and attempt positional cloning of the relevant genes. This has been successfully achieved for several single genes in other fields of inquiry (Probst *et al.*, 1998) but has yet to be carried out for a QTL. Nevertheless, *Sac* and *Qui* (or *Soa*) are attractive candidates for positional cloning because the phenotypic effect of allelic substitutions at these loci is large and this is a major advantage in relating phenotype to genotype. Lush *et al.* (Lush *et al.*, 1995) used categorical assignment procedures (i.e. preferring versus non-preferring) to map *Sac* to a more precise location distal to *D4Mit42*. Their approach assumed that loci in other chromosomal locations had little or no influence on intake of relevant solutions. If such loci exist, however, their contribution to the scores of individual animals could result in mis-classification and errors in estimating the location of *Sac*. Thus, in addition to providing potential new models for investigation, the results of QTL studies of the whole genome will assist the fine-mapping of *Sac* and *Qui* by describing the genetic architecture underlying intake of relevant solutions.

Other QTL sites on chromosomes 4 and 6

As previously noted, Ninomiya *et al.* (Ninomiya *et al.*, 1991) reported the existence of a gene (*dpa*) on proximal chromosome 4 that influenced variation in CTA generalization from D-phe to sucrose in a cross of C57BL/6CrSlc and BALB/cCrSlc strains. Bachmanov *et al.* (Bachmanov *et al.*, 1997) also detected a provisional QTL on proximal chromosome 4 that contributed to variation in two-bottle sucrose intake in their cross. Their data furthermore suggested an important interaction between proximal and distal QTL: demonstration of an influence of allelic variation at the proximal location required the presence of a C57BL6By allele on distal chromosome 4 (and vice versa). They suggested that the proximal QTL was likely to correspond to *dpa*. Aside from our inability to detect a QTL on proximal chromosome 4 using both raw intakes of sucrose and saccharin as well as intakes adjusted for the large effect of *D4Mit42* on distal chromosome 4, analyses of variance provided no indication of an interaction between proximal and distal markers. However, our failure to detect a QTL in this location should not be taken as evidence for or against the existence of the *dpa* locus. We used a different phenotyping procedure than the two earlier studies and the DBA/2J (the strain we crossed with B) and BALB/cCrSlc— the strain Ninomiya *et al.* (Ninomiya *et al.*, 1991) crossed with C57BL/6CrSlc—strains may carry different alleles at the *dpa* locus.

To clarify the possible contribution of the other variables to the experimental outcomes, it would be useful to carry out studies in which the different phenotypes were mapped in the same lineage. In this case, Capeless and Whitney's strain comparison of consumption of a range of concentrations of D-phe and saccharin using the two-bottle test would be helpful in selecting suitable strains for crossing (Capeless and Whitney, 1996). No other locations on chromosomes 4 and 6 reached statistical significance.

Overview

The large effects that *Sac* and *Qui* exert on intake of 'sweet' and 'bitter' solutions are consistent with the existence of genes which play an important role in gustation. As noted, *Sac* may well work via a peripheral site of action, but the gene(s) responsible remains to be identified. As confidence intervals circumscribing these QTL are reduced by implementation of fine-mapping protocols (Darvasi, 1997), both cloning and candidate gene approaches will become practical. One candidate gene, α-gustducin, has not yet been mapped but, given the effects of an α-transducin knockout preparation on intake of both 'sweet' and 'bitter' solutions (Wong *et al.*, 1996), it would be a provocative candidate for either *Sac* or *Qui*, if it is found to reside in the pertinent regions of chromosomes 4 or 6. In the final stages of manuscript completion, another candidate gene has emerged as a plausible candidate for *Sac*. TR1 is a clone of a 7-transmembrane domain protein receptor (Hoon *et al.*, 1999). The sequences it shares with candidate mammalian sensory receptors, its specific expression in taste receptor cells of the tongue and palate and its high topographic selectivity (it is differentially expressed in fungiform versus circumvallate papillae) suggest it as a highly plausible candidate for *Sac*. There is an urgent need to conduct studies in which *Sac* and TR1 are typed in appropriate mapping populations.

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