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Mapping of putative ether-anesthesia resistance gene using C57BL/6J and MSM/Ms mouse strains

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Abstract
Purpose. We attempted to identify the locations of major mouse genes responsible for sensitivity to diethylether (ether) anesthesia, using microsatellite linkage analyses including Quantitative Trait Locus (QTL) analysis.
Methods. To determine the locations of ether anesthesia resistance genes on chromosomes, an ether anesthesia-resistant mouse strain, C57BL/6J (C57BL), and an ether anesthesia-sensitive mouse strain, MSM/Ms (MSM), were used. The sensitivity of mice to ether anesthesia was determined from the latency time required to lose the righting reflex during exposure to 4% ether vapor in air. The (C57BL × MSM) F_1 mice were found to be resistant to ether, showing that the resistant phenotype is genetically dominant. Twelve resistant and 12 sensitive mice were then selected from the 196 backcrossed F_2 mice (F_1 × MSM) at 11–16 weeks of age. Genomic DNA samples were extracted from the tails for mapping ether anesthesia-related genes using microsatellite linkage analyses.
Results. One major putative gene related to resistance to ether anesthesia was restricted in the region 23 to 37 cM from the centromere in chromosome 7 by primary and secondary linkage analyses. The QTL analysis narrowed the position of the gene to 29.0 cM, with a maximum logarithm of odds (LOD) score of 3.03, and it was termed *Etan1* (*ether-anesthesia 1*).
Conclusion. Microsatellite linkage analyses, including QTL analysis, determined the location of the ether-resistance gene, *Etan1*, within a narrow range. Our findings should be helpful for further experiments, such as cloning of the gene governing the sensitivity to ether anesthesia in mice.

Key words Diethylether · Anesthesia resistance gene · Mouse · QTL · Microsatellite marker

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Introduction

Recent genetic research has discovered several genes that are responsible for hereditary diseases and cancers. Further, many of their locations on the chromosome have been identified by the development of linkage analyses using microsatellite markers [1–3], and such genes have been cloned using gene-engineering techniques. Cloned genes are useful for elucidating diseases in molecular biological terms and are helping to increase the understanding of hereditary and incurable diseases. Sensitivity to anesthetics is also considered to be governed by genes, because many mutants with heritable altered sensitivities to general anesthetics have been isolated and analyzed in *Drosophila melanogaster* [4,5] and *C. elegans* [6] strains, as well as mouse strains [7–11].

In most eukaryotic cells, gene recombination takes place by crossing-over between homologous chromosomes in germ cells during meiosis. The probability of recombination is usually proportional to the distance between two genetic loci; thus, the recombination frequency is logically proportional to the physical distance between two genetic loci on chromosomes. Based on this principle, linkage analysis is a method used to determine the distances between several loci of markers and genes on a chromosome. A large number of identified markers dispersed on the chromosomes are utilized as milestones to precisely determine the sites of crossover and infer the locus of the gene of interest. Among them, microsatellite markers [1–3] have been identified at 6000 or more sites on mouse chromosomes [12,13]. We performed linkage analyses utilizing microsatellite markers on chromosomes to determine the sites of genes involved with ether anesthesia, by analyzing the relationships between the latency times until the mice became anesthetized and the genetic patterns of the descendants of ether-resistant and ether-sensitive mice.

Kobayashi [7] reported that mouse strain C57BL/6 (C57BL) was resistant to ether anesthesia compared with other strains, i.e., ddY, ICR, DBA/2, BALB/c, and C3H/He, while we found that the MSM/Ms (MSM) strain was sensitive to ether anesthesia when it was compared with the C57BL strain in the 3.0% ether/air condition [10]. In the present study, we attempted to identify the loci of the genes related to ether-anesthesia resistance using these two mouse strains.

Materials and methods

Animals

C57BL and MSM mice were bred in the animal facility of the Research Institute for Advanced Science and Technology, Osaka Prefecture University. The mice were maintained under normal conditions of light (from 7:00 to 19:00), temperature (24 ± 0.5°C), relative humidity (55 ± 5%), and ventilation (10–12 times · h^{-1}). The mice were given the MF laboratory diet (Oriental Yeast Co., Tokyo, Japan) and sterilized tap water ad libitum. They were used for analysis at the age of 11 to 16 weeks. These conditions and further treatments of mice were in accordance with the Japanese Standards relating to the Care and Management of Experimental Animals.

Check of ether gas concentration

Ether at 1.92 ml, 1.44 ml, and 0.96 ml in a glass desiccator with a volume of 10.6 l in a room controlled at 24 ± 0.5°C and a relative humidity of 55 ± 5% theoretically produces 4.0%, 3.0%, and 2.0% of ether vapor in air after 2 min, respectively. The exact concentrations of ether were measured using a Rikken gas checker (Tokyo, Japan).

Determination of latency time to anesthesia

The latency time to anesthesia is one of the indices used to determine the sensitivity to anesthetics. For exposure to 4.0% ether, 1.92 ml of ether was sprayed via a pipette into the glass desiccator, and then the desiccator was immediately covered with a glass lid with a gum stopper on its top. After the desiccator was kept tightly closed for 2 min, two to six mice were placed inside through the gum stopper hole, and the hole was closed with the stopper again. The latency time required for falling into anesthesia was recorded as the time until the mouse could not show a righting reflex for at least 5 s. All of the anesthetized mice were confirmed to recover from anesthesia. Measurements of latency times were carried out twice for each mouse at intervals of 7 days or more, and the average time for each mouse was considered the latency time. If the values of two trials were significantly different, an additional trial was performed, and the average of the third value and the closer value of either the first or the second time was utilized for further analysis.

In order to examine the significance of the difference in latency times (sensitivities to ether) between two groups, Welch's *t*-test was utilized.

Genotyping of simple sequenced repeats

Different mouse strains (such as C57BL and MSM) often have different lengths of DNA segments (up to several hundred base pairs), consisting of multiple tandem repeats of 2–4 base-pair sequences (for example, $[CA]_n$, and $[AATG]_n$), depending on the repeat number (n), which are termed simple sequenced repeats (SSRs) [2], because these DNA segments can expand or contract with the addition or removal of repeated units with a high frequency [1–3]. Therefore, the difference in the SSR length is useful as a marker for genotyping, that is, determining from which parent strain the part of the chromosomal DNA in question was derived [1–3]. Such varieties of SSR length (or number of repeats) can be detected by the polymerase chain reaction (PCR) (see the next section) method using a specific pair of oligodeoxynucleotides of ca. 20 bases each [1–3]. The pairs of oligodeoxynucleotides utilized for genotyping are called microsatellite markers [1–3].

In the present study, genomic DNA samples were isolated from mouse tails from 196 backcross (F_1 × MSM) progenies (F_2) at 11 to 16 weeks of age for linkage analyses, and PCR was performed to determine the genotype of each mouse for each marker (Fig. 1), according to a procedure described elsewhere [14,15].

Polymerase chain reaction

As shown in Fig. 1, the genotype of the part of the chromosomal DNA in question was determined to be MSM/MSM if only one PCR band could be detected, or C57BL/MSM if two PCR bands could be detected, by marker A or B. PCR amplification of the genomic DNA by a microsatellite marker (or a pair of oligodeoxynucleotides) was performed according to the method described in Okumoto et al. [15]. Briefly, a volume of 10 μl with 50 ng of DNA and 6.6 μM of forward and reverse primers (for each microsatellite marker), along with 1 mM of dNTPs and 0.5 μl of Taq polymerase (Takara, Tokyo), was mixed in a tube at a quantity of 0.6 ml (Quality Scientific Plastics, Petaluma, CA, USA) and then applied to a Program Temp Control System (Model PC-700, ASTEC, Tokyo, Japan). Oligodeoxynucleotides (microsatellite markers) were purchased

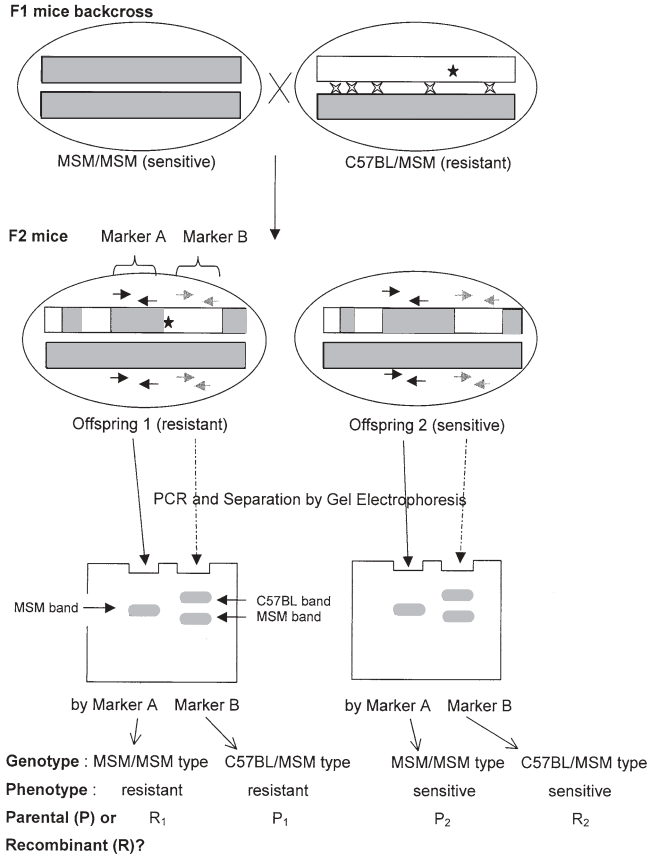

Fig. 1. Principles of genotyping of simple sequenced repeats (SSRs) using the polymerase chain reaction (PCR) method. ▭ Chromosome derived from MSM strain. ▭ Chromosome derived from C57BL strain. ★ Gene related to ether resistance. ☆ Crossover (chromosomal recombination) points in F_1 mice. In the F_1 C57BL/MSM (resistant) mice, the anesthesia-related gene (★) and the C57BL-type marker B (band size longer than that of the MSM-type marker B, as shown in the gel electrophoresis pattern) are usually taken over together to the next generation, as shown in Offspring 1. However, since chromosomal recombination occurs randomly during meiosis, the anesthesia-related gene (★) and the C57BL-type marker B are sometimes taken over to the next generation separately, as shown in Offspring 2. If an offspring mouse had the MSM/MSM type for marker A with the resistant phenotype, it was classified as "recombinant type (R_1)" for marker A. If an offspring mouse had the C57BL/MSM type for marker B with the sensitive phenotype, it was classified as "recombinant type (R_2)" for marker B. If an offspring mouse had the C57BL/MSM type for marker B with the resistant phenotype, it was classified as "parental type (P_1)" for marker B. If an offspring mouse had the MSM/MSM type for marker A with the sensitive phenotype, it was classified as "parental type (P_2)" for marker A. As the distance between a marker and the anesthesia-related gene (★) becomes closer, the rate of the recombinant type ($R_1 + R_2$) compared with the parental type ($P_1 + P_2$) decreases; in other words, the marker and the anesthesia-related gene have a strong linkage

from Research Genetics (Huntsville, AL, USA). The amplification program consisted of 1 cycle of 3 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and finally 1 cycle of 10 min at 72°C. Each PCR product was mixed with 2 µl of loading buffer and electrophoresed on an 8% polyacrylamide gel for 3–4 h at 100 V. The gels were stained with ethidium bromide and, when necessary, silver-stained using a staining kit (Daiichi Pure Chemicals, Tokyo, Japan).

Primary and secondary linkage analyses to determine the gene locus

Primary linkage analysis was performed with backcross progenies of 7 (C57BL × MSM)F_1 females (resistant to ether anesthesia) and MSM males (sensitive to ether anesthesia). Forty microsatellite markers (Research Genetics; see Table 1), which were able to discriminate the length varieties of the SSRs between the C57BL and MSM strains, were selected and used for the primary linkage analysis. The secondary linkage analysis was performed for the candidate region on chromosome 7 determined by the primary linkage analysis by using the 10 microsatellite markers (Table 2).

Statistics of the primary and secondary linkage analyses

As described in the Results, the resistant strain C57BL has a latency time of more than 9 min, whereas the sensitive strain MSM has a latency time of less than 8 min in the 4.0% ether/air condition. For every ether-resistant and sensitive mouse classified by this criterion, the genotype was identified as MSM/MSM or C57BL/MSM by a microsatellite marker, and further, the linkage between the anesthesia-related gene and the microsatellite marker was examined. Under the null hypothesis that there is no linkage between the anesthesia-related gene and the microsatellite marker in analysis, the segregation ratio of the mice would be resistant C57BL/MSM (P_1) + sensitive MSM/MSM (P_2):resistant MSM/MSM (R_1) + sensitive C57BL/MSM (R_2) = 1:1. P_1 and P_2 are the numbers of "parental" types, that is, the number of resistant C57BL/MSM mice and sensitive MSM/MSM, respectively, while R_1 and R_2 are the numbers of "recombinant" types, that is, the numbers of resistant MSM/MSM mice and sensitive C57BL/MSM mice, respectively (Fig. 1, Tables 1 and 2). The stronger the linkage between the gene and the marker, the greater the χ^2 values. With 1 degree of freedom, in the case of a χ^2 value of more than 3.84, the *P*-value is less than 0.05 (5%), and therefore the gene and the marker are considered to have a significantly strong linkage [16]. The χ^2 values were calculated according to the following formula [16]:

Table 1. Primary linkage analysis between sensitivity to diethylether anesthesia and genotypes according to microsatellite markers on the autosomal chromosomes of backcross progenies

Phenotype		Resistant		Sensitive		
Parental or recombinant		R_1	P_1	P_2	R_2	
Chromosome no.	Marker (cM)	MSM/MSM	C57BL/MSM	MSM/MSM	C57BL/MSM	χ^2 Value
1	D1Mit5 (31)	6	6	5	7	0.17
1	D1Mit16 (70)	4	7	8	7	0.62
2	D2Mit7 (18)	4	8	2	10	0.67
2	D2Mit12 (45)	4	8	4	8	0
2	D2Mit25 (80)	4	8	7	4	2.13
3	D3Mit11 (35)	6	6	8	4	0.67
3	**D3Mit21 (39)**	3	9	8	4	**4.17**
4	D4Mitl7 (32)	5	7	6	6	0.17
4	D4Mit7 (36)	3	9	4	7	0.39
4	D4Mit13 (71)	5	7	6	6	0.17
5	D5Nds2 (37)	2	10	2	10	0
5	D5Mit51 (82)	7	5	6	6	0.17
6	D6Mit16 (32)	3	9	5	7	0.67
6	D6Mit14 (71)	6	6	8	4	0.67
7	**D7Mit18 (32)**	2	9	10	2	**9.78**
7	D7Mit14 (72)	7	5	7	5	0
8	**D8Mit11 (3)**	7	4	2	10	**5.26**
8	D8Mit13 (59)	6	5	7	5	0.04
9	D9Mit6 (31)	4	7	6	6	0.39
9	D9Mit18 (66)	4	7	7	5	1.09
10	D10Mit15 (26)	6	5	6	6	0.04
10	D10mit13 (68)	4	7	5	7	0.04
11	D11Mit4 (36)	3	8	6	6	1.09
11	D11Mit10 (59)	4	7	8	3	2.91
12	D12Mit4 (37)	7	5	7	5	0
12	D12Mit181 (53)	7	5	8	4	0.17
13	D13Mit4 (19)	3	8	8	4	3.52
13	D13Mit9 (49)	6	6	7	4	0.39
14	D14Mit4 (12)	5	7	5	6	0.04
14	D14Mit8 (35)	5	7	6	6	0.17
15	D15Mit2 (10)	6	4	3	6	1.32
15	D15Mit17 (30)	4	7	3	8	0.18
16	D16Mit4 (33)	4	7	4	8	0.04
16	D16Mit5 (50)	3	8	7	5	2.13
17	D17Nsd3 (17)	8	4	7	5	0.17
17	D17Mit20 (42)	7	4	6	5	0.18
18	D18Mit14 (23)	8	4	8	4	0
18	D18Mit8 (47)	6	6	8	4	0.67
19	D19Mit12 (20)	5	5	6	6	0
19	D19Mit11 (29)	7	5	6	6	0.17

Numerals under Chromosome no. refer to the chromosome number of mice in which the indicated markers (D1Mit5 to D19Mit11) are located. Numerals in parentheses are distances (cM) from the centromeres. Mice were first classified into groups resistant or sensitive to ether anesthesia and then subgrouped into two genotypes, MSM/MSM and C57BL/MSM, with respect to the marker indicated. χ^2 values were calculated as described in Materials and methods. Markers and χ^2 values in **bold** are mentioned in the text

$$\chi^2 = \left(P_1 + P_2 - P_{\exp}\right)^2 \Big/ P_{\exp} + \left(R_1 + R_2 - R_{\exp}\right)^2 \Big/ R_{\exp}$$

$P_{\exp}$ and $R_{\exp}$ are the expected numbers of the parental-type and recombinant-type mice, respectively, under the the null hypothesis that there is no linkage between the anesthesia-related gene and the microsatellite marker in analysis. Because $P_{\exp} = R_{\exp} = (P_1 + P_2 + R_1 + R_2)/2 = N/2$, where N is the total number of mice, the formula can be simplified as:

$$\chi^2 = \left(P_1 + P_2 - R_1 - R_2\right)^2 \Big/ N$$

Precise determination of the gene locus by quantitative trait loci (QTL) analysis

Quantitative traits of the phenotypes, such as body height and weight, are described by continuous numerals, unlike the phenotypes of qualitative traits, such as

Table 2. Secondary linkage analysis between sensitivity to diethylether anesthesia and genotypes on chromosome 7

Phenotype	Resistant		Sensitive		
Parental or Recombinant Marker (cM)	R_1 MSM/MSM	P_1 C57BL/MSM	P_2 MSM/MSM	R_2 C57BL/MSM	χ^2 Value
D7Mit20 (3)	3	9	9	3	6.00
D7Mit72 (10)	4	8	9	3	4.20
D7Mit273 (20)	2	10	10	2	10.67
D7Nds5 (22)	2	10	10	2	10.67
D7Mit313 (23)	1	10	10	2	12.57
D7Nds1(29)	1	10	10	2	12.57
D7Mit18 (32)	2	9	10	2	**9.78**
D7Nds2 (37)	2	10	10	2	10.67
D7Mit9 (54)	3	8	10	2	7.35
D7Mit14 (72)	7	5	7	5	0.00

cM, genetic distance from centromere of chromosome 7. Other comments are the same as for Table 1

flower color. Sensitivity to anesthetics in mice, especially the latency time to become anesthetized, is a quantitative trait. Genes that govern quantitative traits are called quantitative trait loci (QTL). Prediction of the loci of genes governing the QTL between microsatellite markers is called "QTL analysis" [17–19]. For example, the gene locus of high blood pressure, which is assumed to be heritable, although the loci of the responsible genes are not yet clear at the DNA level, can be determined by QTL analysis.

We first set a null hypothesis (H_0) that the gene related to anesthesia resistance is not linked to a microsatellite marker X, in which case the chromosomal recombination rate (θ) between the gene and marker X is regarded as $\theta_0 = 0.5$ (the maximum value of θ). Then, we set an alternative hypothesis (H_A) that the gene related to anesthesia resistance is linked to microsatellite marker X, with a recombination rate of θ_A ($0 < \theta_A < 0.5$), which reflects the distance between the gene and marker X. A smaller recombination rate (θ_A) means a stronger linkage or shorter distance between the gene and marker X (Fig. 2A). The likelihood (L) that we could obtain the experimental results regarding the separation patterns for parental ($P = P_1 + P_2$) versus recombination ($R = R_1 + R_2$) mice (see Tables 1 and 2) by the marker X under H_0 (in other words, the hypothesis of no linkage) is described as $L(\theta_0)$ or $L(0.5)$, and that under H_A is $L(\theta_A)$. The ratio $L(\theta_A)/L(0.5)$ is called the odds, and $Z(\theta_A) = \log_{10}[L(\theta_A)/L(0.5)]$ is called the logarithm of odds (LOD) score [20]. In the case of no linkage, $L(\theta_A) = L(0.5)$, odds = 1, and therefore the LOD score = 0. The greater $L(\theta_A)$ becomes, the greater the LOD score becomes. In the case of an LOD score greater than 3, in other words, if $L(\theta_A)$ is more than one thousand times greater than $L(0.5)$, the postulated recombination rate θ_A is usually regarded as the proper recombination rate,

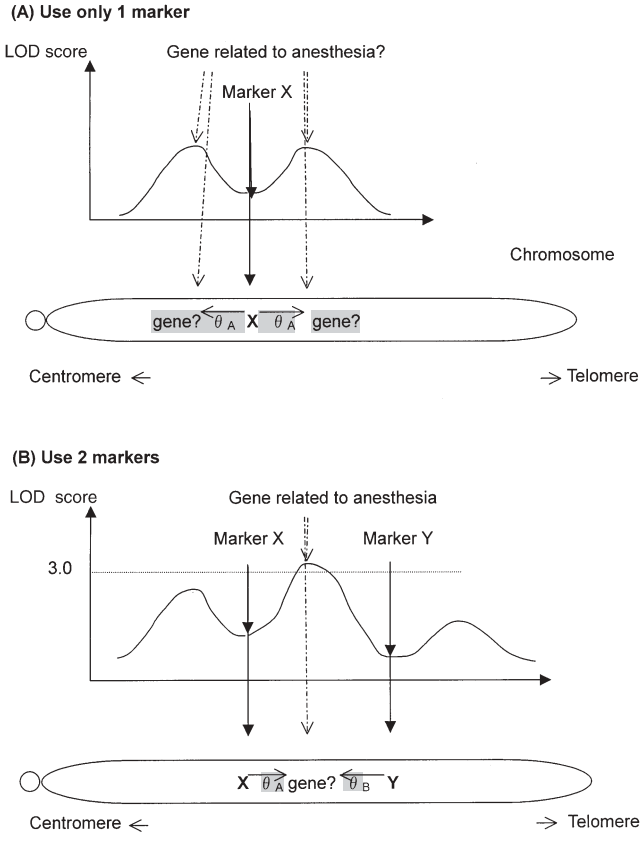

Fig. 2. Simplified view of quantitative trait loci (QTL) analyses. **A** Principle of QTL analysis using one microsatellite marker. **B** Principle of QTL analysis using two microsatellite markers. θ_A is the recombination rate or distance between the anesthesia-related gene and the marker X, and θ_B is the recombination rate or distance between the anesthesia-related gene and the marker Y. $\theta_A + \theta_B$ is equal to the distance between the markers X and Y. QTL analyses using more than three microsatellite markers are available but are not shown here. For details, see Materials and methods

meaning that the gene related to anesthesia is located at a distance of θ_A from the microsatellite marker X (Fig. 2A).

The QTL analysis predicts the candidate(s) of the location(s) of the gene(s) responsible for the QT by calculating each LOD score for marker X by changing the θ values continuously with the help of a personal computer (Fig. 2A). However, to which side (of the centromere or telomere) the gene for the QT is located at the distance θ_A from the marker X remains unknown (Fig. 2A), and therefore more than two markers are necessary (Fig. 2B). In the case of Fig. 2B, another marker Y is also considered, and θ_B is the distance between the gene and the marker Y. If only one peak of the LOD score can be obtained, and the maximum LOD score is more than 3, θ_A and θ_B are regarded as the proper recombination rates between the gene and the microsatellite marker X, and between the gene and the marker Y, respectively, and the gene is located at the distance θ_A from the marker X and θ_B from the marker Y (Fig. 2B). To perform the calculation automatically, the Mapl software developed by Dr. Ukai, University of Tokyo (http://wheat.ab.a.u-tokyo.ac.jp/~ukai/mapl98.html), is convenient. With the use of this software, the curved line of LOD scores at every chromosomal position can be drawn, as shown in Fig. 6.

Results

Strain differences in sensitivity to ether anesthesia

The relationships among the predicted three levels of ether concentration and measured concentrations in the desiccator are shown in Fig. 3. At the expected ether concentrations of 2.0%, 3.0%, and 4.0%, the average concentrations were 2.4%, 3.4%, and 4.0%, respectively. The correlation coefficient (R) was 0.922, and the regression line was $y = 0.083 + 0.817x$, showing the proper correlation between the predicted and the real concentrations of ether.

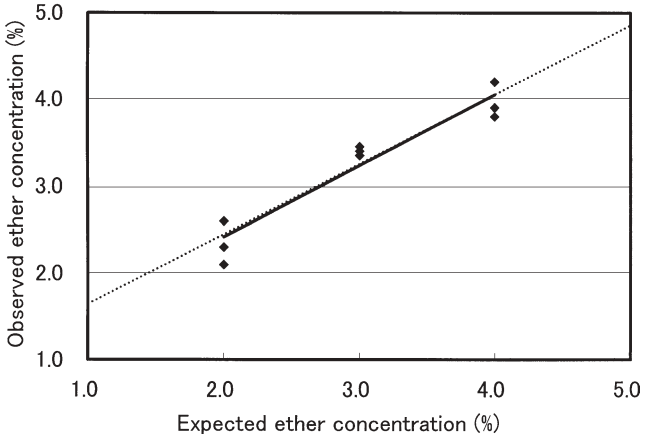

Fig. 3. Correlation of observed concentrations of diethylether and expected concentrations 2 min after spraying of diethylether. Each expected concentration was tested for three trials (◆). The *oblique line* is the linear regression line. For details, see Materials and methods

Inheritance of resistance to ether anesthesia

Figure 4 shows the latency times required for loss of the righting reflex during exposure to 4.0% ether by the C57BL strain (4A), MSM (4B) strain, and (C57BL × MSM)F_1 hybrid (4C) mice. Most C57BL mice (26 out of 30) showed latency times of ≧9 min (Fig. 4A), whereas

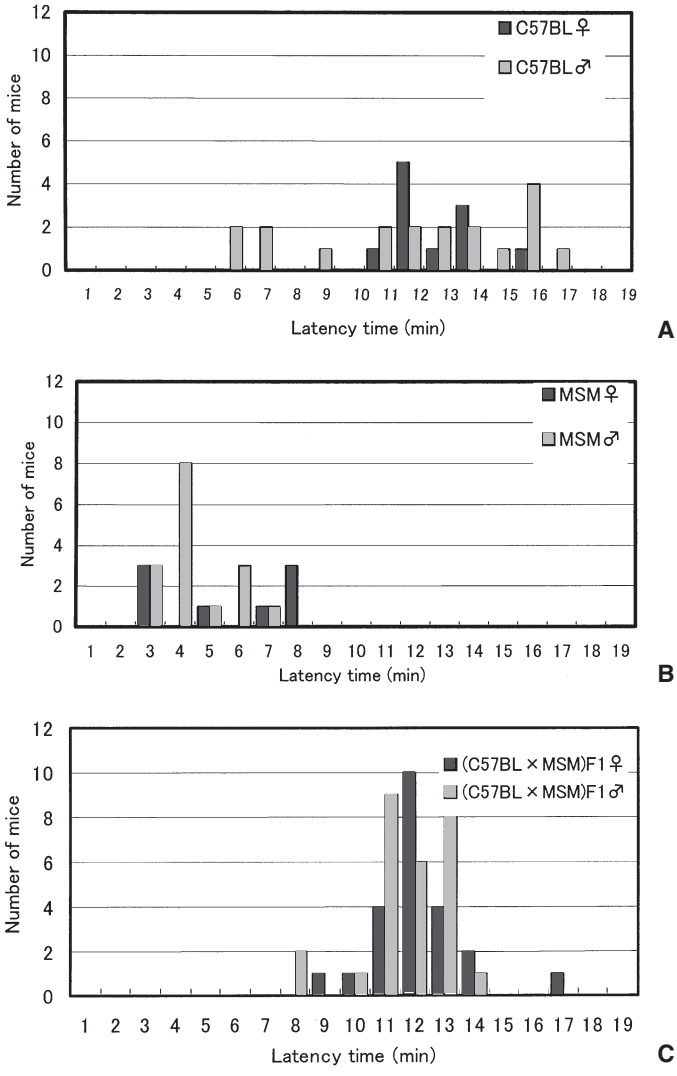

Fig. 4. Distribution of latency times in C57BL (**A**), MSM (**B**), and (MSM × C57BL)F_1 (**C**) mice. In all populations, the distributions of latency times in females (*shaded bars*) and males (*dark bars*) were similar. F_1 (**C**) mice showed a distribution of latency times similar to that of C57BL mice (**A**) rather than MSM mice (**B**)

all MSM mice showed latency times of ≦8 min (Fig. 4B). Therefore, we could confirm that the C57BL and MSM mice revealed the resistant and sensitive phenotypes to ether anesthesia in the 4.0% ether condition, consistent with our previous report [10]. The average latency times of the C57BL strain were 11.9 ± 1.4 min for females (n = 11) and 11.1 ± 2.2 min for males (n = 19); in the MSM strain, they were 3.5 ± 1.2 min for females (n = 8) and 4.8 ± 2.1 min for males (n = 16); and in the F_1 strain, they were 11.2 ± 1.5 min for females (n = 23) and 10.8 ± 1.3 min for males (n = 27). There were no significant differences ($P > 0.05$) between females and males in the three groups (C57BL, MSM, and F_1). There were significant differences ($P < 0.001$) between the C57BL and MSM strains and between the MSM and F_1 strains in the latency times required for ether anesthesia, whereas there was no significant difference between the C57BL and F_1 strains ($P > 0.05$), indicating that resistance to ether anesthesia in C57BL was the dominant trait genetically.

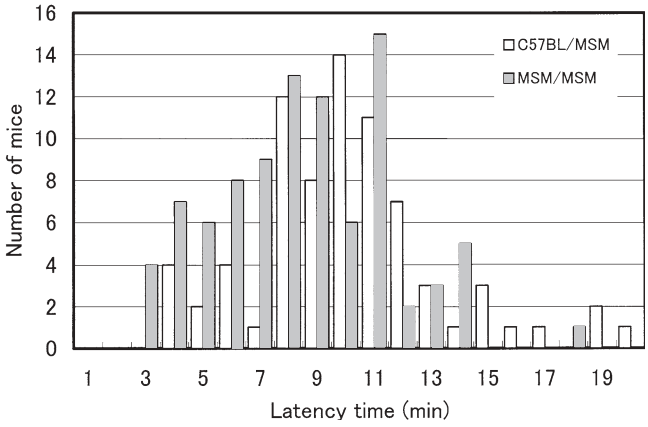

Fig. 5. Distribution of latency times in C57BL/MSM and MSM/MSM genotypic mice by D7Mit313 marker from (MSM × C57BL)F_1 × MSM cross. Distributions of latency times in C57BL/MSM (*open bars*) and MSM/MSM (*shaded bars*) mice were significantly different: 10.6 ± 3.33 and 8.8 ± 2.73 min, respectively. For details, see Results

Linkage analysis for mapping of the anesthesia-related gene

We backcrossed (C57BL × MSM)F_1 with MSM and then randomly selected 12 resistant and 12 sensitive mice from the 169 F_2 mice. The primary linkage analysis was performed with the genomic DNA samples from the 24 mice, using two to three microsatellite markers for every first to 19th chromosome. Table 1 shows the results for analysis of linkage between sensitivity to ether anesthesia and the microsatellite markers on the autosomal chromosomes. The sex chromosome (the 20th) was excluded from this analysis, since no sex-linked genetic pattern was observed (unpublished data). The values of the χ^2 analyses in the 40 kinds of microsatellite markers on 19 chromosomes varied from 0 to 9.78, of which three microsatellite markers, D3Mit21 at 39 cM, D7Mit18 at 32 cM, and D8Mit11 at 3 cM from the centromere, had significant high χ^2 values ($P < 0.05$) of 4.17, 9.78, and 5.26, respectively. The microsatellite marker (D7Mit18) had an extreme χ^2 value, 9.78, indicating that the single major gene related to ether anesthesia should be located near D7Mit18 on chromosome 7, whereas other genes related to ether anesthesia are located near D3Mit21 on chromosome 3 and D8Mit11 on chromosome 13.

The secondary linkage analyses for the region including D7Mit18 were carried out with nine additional microsatellite markers. Table 2 shows the results of the further analyses of chromosome 7. The maximum values of 12.57 by the χ^2 test were recorded for the two markers, D7Mit313 (23 cM) and D7Nds1 (29 cM). Therefore, the position of the major gene can be reduced to the region between D7Mit313 (23 cM) and D7Nds1 (29 cM) on chromosome 7. Histograms of the latency times of anesthetized mice, which were classified into C57BL/MSM and MSM/MSM by the marker D7Mit313, are shown in Fig. 5, and our results indicate that the marker is well linked to the anesthetic phenotype, since the latency times of C57BL/MSM (□) are distributed in the long-time region (right, resistant, 10.6 ± 3.33 min) and those of MSM/MSM (■) are distributed in the short-time region (left, sensitive, 8.8 ± 2.73 min), and the difference in latency times between C57BL/MSM and MSM/MSM is significant ($P < 0.01$ by Welch's t-test).

Figure 6 shows the drawing using the QTL analysis software Mapl. The LOD scores for the microsatellite DNA markers and between markers were calculated at each distance at 0.1-cM intervals. The results showed that the LOD scores for D7Mit72 (10 cM), D7Mit313 (23 cM), and D7Nds2 (37 cM) on chromosome 7 were 0, 2.25, and 1.02, respectively (Fig. 6). However, the locus that gave the maximum LOD score (3.03) was found to reside at 29.0 cM between the D7Mit313 and D7Nds2 markers (Fig. 6) close to D7Nds1. The score of 3.03 at 29.0 cM was slightly over the statistical limit of 3.00 (see Materials and methods). Heritability was also calculated as 0.79 with the Mapl software at the 29.0 cM locus. Heritability is defined as the ratio of [genetic effects to the phenotype]/[whole (genetic + environmental) effects to the phenotype] [21–23]. Since 0.79 was close to the maximum value of 1.00, the locus (29.0 cM) was considered to mainly govern ether-anesthesia resistance with a contribution of ca. 80%; however, there is still the possibility that other factors or other genes on other loci may also contribute to the

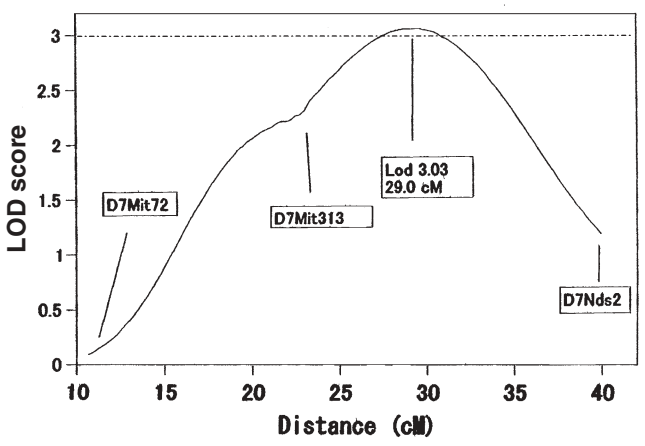

Fig. 6. Logarithm of odds (LOD) scores for chromosome 7 with D7mit72, D7mit313, and D7ds2, by QTL analysis using Mapl software. The *horizontal axis* shows the chromosomal distances (cM) from the centromere, and the *vertical axis* shows the LOD score at each distance. The positions of the three (D7Mit72, D7Mit313, and D7Nds2) are indicated, and the point (29.0 cM) with the maximum LOD score (3.03) is also indicated. *Etan1* would exist at the locus

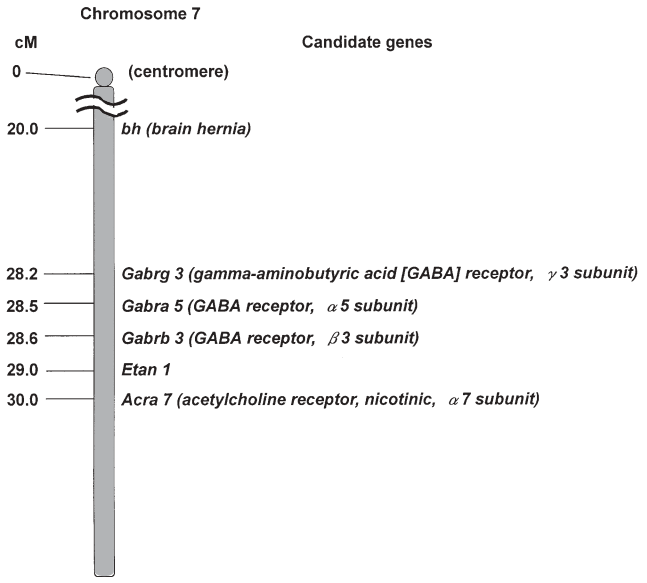

Fig. 7. Location of candidate genes considered to be related to neuronal functions and related to general anesthesia on chromosome 7 from 20 to 30 cM. For details of the candidate genes described, see Discussion

phenotype (environmental effects) with a contribution of ca. 20%. Therefore, we named this novel locus, where the major gene related to ether anesthesia resistance was located, *Etan1* (*ether anesthesia 1*).

Discussion

Volatile anesthetics probably exert an influence at many locations of the signal transfer between neurons, which consist of ion channels and receptors in the neuromembrane, as well as second messengers, enzymes, protein kinase C, and functional proteins in the cytoplasm. However, it has not yet been established which parts of the signal transfer are the targets of general anesthetic agents.

The *Etan1* gene that we identified might encode the ether target gene. The following four known genes have been identified near the region 29.0 cM on mouse chromosome 7: *Gabrg3* [gamma-aminobutyric acid (GABA) receptor, γ3 subunit] at 28.2 cM, *Gabra5* (GABA receptor, α5 subunit) at 28.5 cM, *Gabrb3* (GABA receptor, β3 subunit) at 28.6 cM, and *Acra7* (acetylcholine receptor, nicotinic, α7 subunit) at 30.0 cM (Fig. 7).

One or more of the three receptors, *Gabrg3*, *Gabra5*, and *Gabrb3*, might be the candidate(s) of ether target(s) encoded by *Etan1*, because their locations are very close to 29.0 cM. GABA is a major inhibitory neurotransmitter that opens chloride channels through actions on the GABA receptor, and Franks and Lieb [24] hypothesized that anesthesia is induced by enhancement of the function of GABA receptors by binding anesthetics to GABA receptors. Mutations of GABA receptor gene(s) in the C57BL would reduce the amount and function of the GABA receptor and might offer the mice the resistant phenotype to ether anesthesia.

Several important anesthetic experiments using knockout mice have been performed. Homanics et al. [25] studied mice lacking a functional α6 gene of the gamma-aminobutyric acid type A receptor ($GABA_A$-R) and found that sleep time response to an injection of pentobarbital, as well as loss of righting reflex and response to tail-clamp stimulus following anesthesia with volatile anesthetics, did not differ between wild-type and knockout mice. Further, Quinlan et al. [26] reported that the absence of the β3 subunit of $GABA_A$-R appears to attenuate the obtunding effects of midazolam and etomidate, but does not alter those of pentobarbital, enflurane, or halothane, suggesting that these anesthetic agents produce hypnosis by different specific molecular mechanisms.

Another candidate for *Etan1* is Acra7 at 30.0 cM on chromosome 7. Acetylcholine (ACh) is a major excitatory neurotransmitter that opens cation channels through the nicotinic acetylcholine (nACh) receptors. Relationships between anesthetics and nACh receptors have been reported in several studies, notably by Raines et al. [27], who found that the nACh receptor is inactivated by clinical concentrations of general anesthetics. Usually mutations of a gene reduce the function of the gene product, and therefore, the MSM mice

might have the mutation of the nACh receptor gene which reduces the function of the nACh receptor, causing hypersensitivity to ether anesthesia.

Different anesthetics may have their own targets. Interestingly, Nakao et al. [11] showed that the candidate of the gene relating to hypersensitivity to enflurane anesthesia is located at the *Tyr* locus (44 cM on chromosome 7) in mice. Simpson et al. [28] also found a genetic region, *Lorp1*, in mice that specifies the sensitivity to propofol at 44 cM between D7Mit90 and D7Mit184 of chromosome 7. However, those regions (both are 44 cM) are far from the *Etan1* region that we found, which was 29 cM on chromosome 7 (Fig. 6), indicating that neither *Tyr* nor *Lorp1* is related to the region we analyzed. In order to determine additional genes related to anesthesia, it is important to conduct research using many combinations of ether anesthesia-resistant and -sensitive strains. Nevertheless, the present combination of MSM and C57BL strains showed the largest χ^2 values (see Materials and methods) among all strains that we have tested up to this time.

There is also the possibility that *Etan1* encodes a novel gene related to ether anesthesia, different from GABA receptors or ACh receptors. Further cloning and analysis of the genes at the 29.0 cM locus are necessary to completely elucidate the mechanisms of diethylether anesthesia.

Other genes related to anesthetics (so-called minor genes) may exist, at least in the two loci (D3Mit21 and D8Mit11), because the χ^2 values at these loci were rather high (4.17 and 5.26, respectively; see Table 1). At the D3Mit21 locus, the gene with similar properties to *Etan1* might exist. However, the putative gene on D8Mit11 on chromosome 8 would have different properties from the *Etan1* which confers the resistant phenotype on the MSM/MSM mice and confers the sensitive phenotype on the CL57BL/MSM mice, because it showed the reverse pattern from the other two markers; that is, most of the resistant mice had the sensitive marker genotype (MSM/MSM), and most of the sensitive mice had the resistance one (CL57BL/MSM).

Although there is no evident relationship between inheritance and sensitivity to anesthetics at present in humans, individual susceptibility to anesthesia may be governed by the genetic background of each person, as in mice. Thus, our methods of predicting anesthesia-related gene(s) may be applied to humans, helping to determine the sensitivities of patients to anesthetics.

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