Genetic analysis of inferior nurturing ability in RR mice

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Females of the inbred mouse RR strain have a limited ability to nurture their offspring, and frequently the young die during rearing. Quantitative trait locus analysis was carried out on the F₂ progeny produced from a genetic cross between RR and KK, a strain of normal nurturing ability, to elucidate the putative genetic basis governing certain aspects of the inferior nurturing ability in the RR strain. One hundred and ninety-two F₂ female mice were mated with C57BL/6J males. After the number of newborns was adjusted to six per dam, nurturing ability was evaluated on the basis of litter

weight at days 7, 12 and 21 after parturition. The results showed that 147 of the 192 F₂ dams were able to rear all six pups, although the litter weight varied considerably among litters. Significant evidence for linkage was identified on chromosome 5 near the microsatellite marker of *D5Mit161* for litter weight at 12 days after parturition. The R allele (RR strain) at this locus was recessive to the K allele (KK strain), and reduced the litter weight. It is suggested that this locus is one of the heritable components that determine the inferior nurturing ability of RR mice.

Introduction

Defects in genes related to reproductive ability may lead to reproductive abnormalities, as illustrated by spontaneously occurring and engineered mutant mice (Doolittle *et al.*, 1996; Nishimori and Matzuk, 1996). In general, these mutants are useful for revealing the genetic bases of reproduction. However, the mutant phenotypes are often developed beyond the normal physiological range and therefore do not always reflect the common aspects of the traits. Reproductive traits, such as ovulation rate, embryonic mortality, litter size, duration of gestation and birth interval, are quantitative traits that are controlled by multiple genes under the influence of environmental stimuli (Land, 1984). Quantitative trait loci (QTL) that control some of these traits have been mapped in cattle, pigs and rats (Rathje *et al.*, 1997; Wilkie *et al.*, 1999; Zidek *et al.*, 1999; Kirkpatrick *et al.*, 2000).

Maternal nurturing ability is the capacity to raise and protect offspring through lactation. Normal nurturing requires an adequate interaction between the mother and the young that comprises complex physiological and behavioural processes (Nowak *et al.*, 2000). Inadequate mother–young interaction or inappropriate maternal care of the offspring during the neonatal period leaves neonates vulnerable and their mortality increases.

Inbred mouse RR mothers have a limited ability to nurture their pups but possess no other gross defects with regard to reproductive performance (Festing, 1996; J. Suto, H. Yamanaka and K. Sekikawa, unpublished). Although pregnancies normally continued to term, the newborn young had a low rate of survival until weaning (weaning rate was 69.6% with respect to the number of offspring, and

50% of dams lost pups). Although in depth behavioural studies have not been conducted, RR females do not exhibit overt maternal neglect. Thus, it was hypothesized that the underlying defect is related to poor nutritional support (that is, poor lactation) rather than a behavioural defect.

The aim of the present study was to identify using QTL analysis the gene or genes that control the inferior nurturing ability in RR mice.

Materials and Methods

Mice and F₂ production

All mice used in the present study were of inbred strains. KK females and C57BL/6J males were purchased from CLEA JAPAN Inc. (Tokyo). The RR strain had been maintained in the authors' laboratory, and consisted of more than 125 generations of brother–sister mating. The RR strain was derived from Japanese fancy mice and was obtained from a dealer in Sendai, Japan (Festing, 1996). The KK strain was established in Japan by K. Kondo (Festing, 1996) as a diabetic strain with moderate obesity; however, because of the maturity onset nature of the diabetes and obesity, the influence of obesity and diabetes on fertility at younger ages seems to be negligible, particularly in female mice. Hereafter, we define the RR strain as having R alleles, and the KK strain as having K alleles, throughout the genome.

KK females were crossed with RR males to produce KK × RR F₁, which were intercrossed to produce the KK × RR F₂. On the basis of abdominal expansion, pregnant F₁ mice were housed individually to produce F₂ mice. All F₂ females were weaned at day 30 after birth, and three mice were housed together in each cage.

All mice were maintained in a temperature-controlled

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($22 \pm 3^\circ\text{C}$), specific-pathogen-free room, with a regular light cycle of 12 h light:12 h dark and a relative humidity of 50%. Rodent pellet chow (CE-2; CLEA Japan Inc.) and tap water were available *ad libitum*.

Evaluation of nurturing ability

As nurturing ability is a difficult parameter to measure, litter weight was used as an indirect, but reliable, measurable characteristic in this study. The use of litter weight as an indicator of nurturing ability is justified as follows. (i) The total body weight of a litter has been used as an indirect measure of a mother's milk production. Growth of pups has been shown to be attributable purely to milk obtained from the dam up to day 12 after birth (Falconer, 1947). (ii) The nursing ability of a mother can be measured by the body weight of her young at day 12 after birth, subsequent to the standardization of the litter size at birth (Nagai, 1975; Nagai *et al.*, 1978).

Three virgin F_2 female mice had been placed together per cage until day 70 of age, and then a C57BL/6J male of the same age was introduced into each cage. The pregnant F_2 females were then caged individually. On the day of delivery, pups were culled randomly or were supplemented with pups from other litters of the same date of birth, to an average of six pups per dam. Nurturing ability was evaluated on the basis of the total litter weight of six pups at days 7, 12 and 21 after birth. Low litter weight was considered an indication of inferior nurturing. An analysis was carried out on dams ($n = 147$) that reared all six pups successfully and a second analysis was carried out on all dams ($n = 159$) regardless of whether pups were lost during rearing. In the second analysis, because the microsatellite genotypes could not be examined on the 12 dams that lost some or all of the pups, genotypes are labelled as missing data.

Microsatellite genotyping

The genotypes of a total of 75 (69 autosomal and 6 X-linked) microsatellite markers were determined (Table 1). Microsatellite sequence length polymorphism was detected by electrophoresis subsequent to polymerase chain reaction (PCR). Most microsatellite primers were purchased as MapPairs (Research Genetics, Huntsville, AL), whereas others were synthesized. Amplification was carried out with a Takara PCR thermal cycler MP (Takara Biomedicals, Tokyo) under the following conditions: one cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 45 s; one cycle at 72°C for 7 min. All PCR products were subjected to electrophoresis on 10% (w/v) polyacrylamide gels and identified by ethidium bromide staining.

Quantitative trait locus (QTL) mapping and statistical data analysis

Subsequent to the measurement of phenotypic variables and microsatellite genotyping, QTL analysis was carried out with the Mapmaker/EXP version 3.0b and the Mapmaker/

QTL version 1.1b computer programs (Lander *et al.*, 1987). The chromosomal region with a logarithm of odds (LOD) score of more than 4.3 (threshold of statistical significance at $\alpha = 0.05$) was recognized as significant linkage, and the region with a LOD score between 2.8 and 4.3 was recognized as suggestive of linkage (Lander and Kruglyak, 1995). The α level for suggestive linkage implies the expectation that there will be one false positive in a genome-wide search. The mode of inheritance in each QTL was determined by comparison of the dominant, recessive and additive models given by the Mapmaker/QTL program, in which a LOD score of 1.0 or a greater difference was used as the criterion. The statistical difference of means among the three genotypes partitioned by the closest marker that flanks the putative QTL was analysed by one-way ANOVA.

Results

Breeding results in parental strains, F_1 and F_2

The breeding data on parental RR, F_1 and F_2 mice, together with (female RR $\times$ male KK) F_1 data for comparison, are shown (Table 2). According to the breeding data on KK mice from more than 500 litters provided by CLEA JAPAN, the weaning rate is 97.0%, and the average litter size is 6.2 (litter size was culled randomly to eight pups per dam). The data on parental RR were based on the breeding data compiled in the authors' laboratory, and the number of pups was culled to six at the day of birth. Reciprocal F_1 crosses were made (culling was not carried out) and the results of both types of F_1 dams are presented. Although the litter size in F_1 was twice as large as that in the parental RR and KK strains, and no adjustment of the number of pups was made, the F_1 mice exhibited reasonably good nurturing ability. Thus, inferior nurturing ability is inherited on an overall recessive mode. The F_1 cross of KK females $\times$ RR males was used to generate F_2 animals. For the F_2 analysis, the number of pups was adjusted to six per dam at birth.

Evaluation of nurturing ability in F_2 mice

Of 192 F_2 females mated with C57BL/6J males, 165 gave birth. The average litter size was 8.5 ± 2.9 (mean $\pm$ SD, range 0–15). The number of pups was adjusted to six per dam by culling of the extra pups in most litters (136 of 165 litters, 82.4%). Of the 165 dams, six died accidentally during rearing, and 12 lost some or all ($n = 1$ –6) of their pups during rearing. The number of days from the introduction of a C57BL/6J male to parturition was 41.0 ± 10.0 (mean $\pm$ SD, range 29–73 days).

The litter weights of six pups from 147 litters (in which all six pups were reared) on days 7, 12 and 21 are presented as scatter plots (Fig. 1). The means $\pm$ SD of the litter weights are as follows: 31.49 ± 3.59 g (range 19.61–39.6) on day 7, 45.84 ± 6.27 g (range 24.23–58.27) on day 12 and 71.89 ± 8.49 g (range 48.11–88.40) on day 21.

Table 1. Microsatellite markers used and their chromosomal position*

Marker	Position (cM)	Marker	Position (cM)	Marker	Position (cM)
Chromosome 1		Chromosome 8		Chromosome 14	
D1Mit211	15	D8Mit58	1	D14Mit11	0.7
D1Mit10	56.6	D8Mit205	30	D14Mit64	22
D1Mit102	73	D8Mit267	43	D14Mit94	50
D1Mit16	87.2	D8Mit245	72	Chromosome 15	
D1Mit291	101.5	Chromosome 9		D15Mit136	14.2
Chromosome 2		D9Mit90	9	D15Mit63	29.2
D2Mit182	38.3	D9Mit229	28	D15Mit190	52.8
D2Mit300	50.3	D9Mit107	40	D15Mit35	61.7
D2Mit194	81.4	D9Mit182	55	Chromosome 16	
D2Mit346	91.8	Chromosome 10		D16Mit57	21.5
Chromosome 3		D10Mit123	4	D16Mit139	43.1
D3Mit224	22	D10Mit183	17	Chromosome 17	
D3Mit184	28	D10Mit95	51	D17Mit164	2.8
D3Mit29	45.2	D10Mit151	69	D17Mit176	22.5
Chromosome 4		Chromosome 11		D17Mit93	44.5
D4Mit1	6.3	D11Mit229	14	Chromosome 18	
D4Mit111	21.9	D11Mit86	28	D18Mit177	20
D4Mit251	66	D11Mit219	43	D18Mit149	24
Chromosome 5		D11Mit124	57.8	D18Mit152	37
D5Mit200	36	D11Mit61	70	D18Mit162	50
D5Mit240	59	Chromosome 12		Chromosome 19	
D5Mit161	70	D12Mit136	13	D19Mit68	6
D5Mit221	78	D12Mit190	28	D19Mit55	53
Chromosome 6		D12Mit158	38	Chromosome X	
D6Mit116	5.5	D12Mit28	52	DXMit89	3
D6Mit123	29	Chromosome 13		DXMit166	15.5
D6Mit261	37	D13Mit207	9	DXMit172	48.7
Chromosome 7		D13Mit221	30	DXMit149	50
D7Mit76	3.4	D13Mit110	47	DXMit38	53.2
D7Mit232	26.8	D13Mit78	75	DXMit121	67
D7Mit253	52.8				
D7Mit259	72				

*Chromosomal position was retrieved from the Mouse Genome Database (MGD) on 20 November 2000.

Fig. 1. Scatter plots of litter weight (total weight of six pups) in 147 F_2 litters produced from a genetic cross between RR and KK mice at days 7, 12 and 21 after parturition.

QTL analysis

Seventy-five microsatellite markers (Table 1) were genotyped in all F_2 mice, resulting in an average spacing of 21.3 (1600/75) cM between markers. Thus, the marker density may have been insufficient to detect QTL in chromosomal regions with widely spaced markers. These regions should be analysed in a future analysis.

In an analysis of the 147 dams that reared six pups, statistically significant evidence for linkage was identified on chromosome 5 with a LOD score of 4.5 for litter weight at day 12, near the microsatellite marker of *D5Mit161* (Table 3, Fig. 2). This locus explained 13.7% of the F_2 variance, with an additive effect of -3.18 g total litter weight per R allele. With regard to the litter weight at day 21, this locus showed suggestive evidence for linkage, with a LOD score of 4.0. The R allele at this locus was recessive to the K allele, and reduced the litter weight (Table 4), consistent with the results of the F_1 intercrossing. The gene symbol *Naq1* (nurturing ability QTL

Table 2. Comparison of breeding data in dams from RR, F₁ and F₂ mice

Dams	Number of litters	Number of pups born	Number of pups weaned	Weaning rate (%)	Average litter size
RR*	83	553 (445)	287	64.5	6.7
(KK female × RR male) F ₁	20	224	223	99.6	11.2
(RR female × KK male) F ₁	8	86	85	98.8	10.8
F ₂	165	1410 (990**)	914	92.3	8.5

*Data were compiled only from litters with more than five pups.

**Number of pups was culled to six per dam at birth (number of pups after culling is indicated in parentheses).

Table 3. Identification of quantitative trait loci (QTL) for litter weight

Chromosome	Closest marker	Traits	LOD score	Variance explained (%)	Gene symbol
5	<i>D5Mit161</i>	Litter weight at 12 days	4.5	13.7	<i>Naq1</i>
5	<i>D5Mit161</i>	Litter weight at 21 days	4.0	11.7	
9	<i>D9Mit90</i>	Litter weight at 21 days	3.2	9.6	

Naq1: nurturing ability QTL number 1.

Fig. 2. Identification of nurturing ability quantitative trait locus (QTL) (*Naq1*) on mouse chromosome 5. Logarithm of odds (LOD) scores for litter weight are given at days 7 (○), 12 (□) and 21 (△) after birth. Significant linkage was identified for litter weight at day 12, and suggestive linkage was identified for litter weight at day 21. The lines were based on a free genetic model given by constrained genetic models of the Mapmaker/QTL program. The horizontal dotted line (LOD score 4.3) indicates the threshold of statistical significance ($P = 0.05$).

number 1) was assigned to this locus. The *Naq1* is located near the genes encoding zona pellucida protein 3 (*Zp3*), zonadhesin (*Zan*), and a disintegrin and metalloproteinase domain 1a (*Adam1a*) (Kozak and Stephenson, 1998). *Zp3*

and *Adam1a* have roles in fertilization of the oocyte (Doolittle et al., 1996; Wolfsberg and White, 1996), whereas *Zan* is expressed in haploid spermatids (Hardy and Garbers, 1995). Another locus was identified on proximal chromosome 9 for pup weight at day 21, near the microsatellite marker of *D9Mit90* with a LOD score of 3.2. Although the linkage was merely suggestive, the R allele at this locus was dominant to the K allele and reduced the litter weight (Tables 3 and 4). Although the levels of linkage were not identified for other traits, such as litter size and days to parturition, the influence of *Naq1* on these traits was also examined. The *Naq1* genotype was found to be associated with litter size, and homozygotes produced significantly fewer pups: KK: 9.1 ± 2.8 ($n = 38$); KR (heterozygote): 9.2 ± 2.3 ($n = 75$); RR: 7.7 ± 3.0 ($n = 34$) (means $\pm$ SD, $P < 0.03$ on a single point statistical basis (Lander and Kruglyak, 1995)). Therefore, the mode of inheritance of the R allele at the *Naq1* locus is recessive to the K allele and reduced the number of newborn pups.

In the alternative analysis of 159 dams (the above-mentioned 147 dams plus 12 dams that lost some or all of their pups), statistically significant evidence for linkage was identified on chromosome 5, with a LOD score of 4.7 for litter weight at day 12, near the microsatellite marker of *D5Mit161*. This locus explained 22.8% of the F₂ variance, with an additive effect of -5.62 g total litter weight per R allele. This locus showed suggestive evidence for linkage on litter weight at day 21, with a LOD score of 4.2. However, a locus on chromosome 9 did not show even suggestive evidence for linkage at any points of measurement. Thus, when the litter weight data from dams that lost pups were included in the analysis, the LOD score on the *Naq1* (chromosome 5) locus became higher, which explains the larger variance compared with that of the chromosome 9 locus.

Table 4. Results of ANOVA

Marker	Traits	Genotype			Nominal <i>P</i> value*	Mode of inheritance of the R allele
		RR	RK	KK		
<i>D5Mit161</i>	Litter weight at 12 days	41.9 ± 7.1 (34)	46.5 ± 5.4 (75)	48.1 ± 5.6 (38)	<i>P</i> < 0.00005	Recessive
<i>D5Mit161</i>	Litter weight at 21 days	66.7 ± 10.2 (34)	72.9 ± 7.0 (75)	74.5 ± 7.7 (38)	<i>P</i> < 0.0002	Recessive
<i>D9Mit90</i>	Litter weight at 21 days	69.7 ± 8.6 (32)	70.6 ± 8.8 (75)	76.1 ± 6.4 (40)	<i>P</i> < 0.0007	Dominant

Values in parentheses indicate the number of mice.

*The genome-wide threshold for statistical significance in an *F*₂ intercross is a LOD score of 4.3, corresponding to a *P* value of 5.2×10^{-5} (Lander and Kruglyak, 1995).

Discussion

Heritable factors are thought to determine the predisposition to low fertility (Land, 1984). Females of the RR strain are apparently inferior in their ability to nurture their offspring (J. Suto, unpublished). In about 50% of RR litters, some of the newborn young do not survive to weaning and there is an overall 64.5% weaning rate. This low weaning rate seems to be a consequence of poor maternal nurturing ability rather than high mortality of the RR pups, although this has not been validated conclusively. RR dams tend to provide poor nurturing of *F*₁ pups produced from sires of other inbred strains; however, dams from other inbred strains mated with an RR sire nurtured young normally. In the *F*₂ generation in the present study, six dams (3.6%) died while rearing their young, indicating that it is a considerable burden for them to nurture their offspring. In contrast, none of the 27 non-pregnant females died. Furthermore, the litter weight varied considerably among the litters but weight differences among individual pups were not so prominent. RR mice were not inferior to other mouse strains with regard to other reproductive traits, such as litter size, sex ratio or days required for the first delivery. Therefore, RR mice are a particularly useful animal model for investigating the genetic cause of the impaired maternal nurturing, because the trait can be examined without having to take into consideration other traits that may influence the nurturing ability. For clarification of the limited nurturing ability, further behavioural studies will be required. However, the underlying defect is likely to be related to poor lactating ability rather than a behavioural defect, because RR females do not exhibit overt maternal neglect. Poor lactating ability may be the single major defect in RR mice but it is also possible that pups of the RR strain have some inherent congenital defects. It is hoped that this possibility can be tested in the near future by cross-fostering between RR and KK strains, and postmortem examination of pups.

In the present study, a significant QTL was identified for nurturing ability (total body weight of 6 pups at day 12 after

birth) on chromosome 5, and there were two possible linkages on chromosome 5 and chromosome 9 (both for litter weight at day 21). In the region neighbouring the QTL on chromosome 5, there are loci that pertain to reproduction (Kozak and Stephenson, 1998). The first locus contains zona pellucida protein 3 (*Zp3*). *Zp3* knockout mice have been established and the females were found to be infertile (Rankin *et al.*, 1996). Therefore, the possibility that *Zp3* plays a role in maternal behavior remains unanswered. The second locus is for zonadhesin (*Zan*), which encodes a sperm membrane protein (Hardy and Garbers, 1995). The third locus is for a disintegrin and metalloproteinase domain 1a (*Adam1a*, fertilin alpha), a member of the *Adam* gene family. There are several ADAMs proteins that operate during fertilization and development (Wolfsberg and White, 1996). Although *Zp3*, *Zan* and *Adam1a* are involved in certain aspects of fertility, there is no evidence to indicate that they affect the maternal ability to nurture.

Although no separate QTLs responsible for litter size were identified in the present study, the fact that the genetic locus responsible for nurturing also has an influence on litter size indicates that these distinct variables share a common basis. Furthermore, the R allele at the *Naq1* locus was recessive for both nurturing ability and litter size.

Genetic studies on several knockout mice, such as studies on *fosB* (FBJ osteosarcoma oncogene B, chromosome 7) (Brown *et al.*, 1996), *Prlr* (prolactin receptor, chromosome 15) (Lucas *et al.*, 1998) and *Oxt* (oxytocin, chromosome 2) (Nishimori *et al.*, 1996) have demonstrated that the maternal behaviour of nurturing is regulated genetically. These studies indicate that numerous genes may influence maternal behavior. The fact that nurturing ability in RR mice is not as limited as in the abovementioned knockout mice may lead to difficulties in defining the major reason for poor nurturing.

The fact that a higher LOD score was obtained on the *Naq1* (chromosome 5) locus when the analysis included dams that had lost pups increases the probability of the existence of the *Naq1* QTL. The existence of the QTL was

confirmed in a second analysis that reflects the defective nature of the RR, that is, that RR female mice wean a smaller percentage of pups and litters. However, the analysis that included dams that lost pups did not detect even a suggestive linkage on chromosome 9.

The results of the present study indicate that there are genetic components, as yet unidentified in other mutant mouse systems, which control poor nurturing ability in the RR strain.

Identification of loci that control reproductive behaviour will lead to better understanding of maternal behaviour and will assist in the successful reproduction of laboratory animals and economically important livestock.

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