A Novel Growth Retardation and Abnormal Gonad Morphology Locus on Mouse Chromosome 4

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Mutant mice exhibiting a growth retardation phenotype arose spontaneously in the inbred NC/Sgn mouse strain. The mode of inheritance of this mutation was autosomal recessive, and I named this mutation growth deficit (gd). The gd locus was mapped to the proximal part of chromosome 4, between microsatellite markers D4Mit139 and D4Mit178. Histologic abnormalities were detected in the mutant testis and ovary. Degeneration and/or necrosis were found in the seminiferous epithelium, particularly in the spermatocytes; therefore, mutant males were thought to be sterile owing to defective spermatogenesis. Mutant ovaries were generally atrophied. Necrosis of granulosa cells and increased number of atretic follicles were remarkable. The gd locus is suggested to be syntenic to human chromosome segment 9q32–q34, to which no similar mutations had been mapped. Although the molecular nature of this mutation is unclear, gd promises to make future contribution to relevant diseases in human beings.

Key words: abnormal gonad morphology, genetic mapping, growth retardation

Mutant mice exhibiting growth retardation phenotype arose spontaneously in the inbred NC/Sgn mouse strain. Mutants were usually discriminated from normal littermates on and after 2–3 weeks of birth. Mutants showed wasted phenotype with the advancement of age, and most mutants survived for a maximum of 3 months empirically. The mode of inheritance of the mutation was suggested to be autosomal recessive; therefore, I named this mutation growth deficit (gd). A difference in body size in NC-gd/gd mice usually became apparent on and after 2–3 weeks of age and gradually became wasted, and therefore, mating between homozygous (gd/gd) males and females was impeded. Thus, the gd mutation has been maintained through mating between putative heterozygous (gd/+ ) males and females.

All mice were maintained in a specific pathogen-free facility with a regular light cycle and controlled temperature and humidity. Food and water were freely available throughout the experimental period. All the animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of NIAS.

Genetic Mapping of Mutant Gene Locus

In order to perform chromosomal mapping of the mutant gene locus, a total of 369 F2 mice were produced and analyzed. Three hundred and five F2 mice were produced by intercrossing between (C57BL/6J × NC-gd/gd) F1-gd/+ and 64 F2 mice were produced by intercrossing between (C57BL/6J × NC-gd/gd) F1-gd/+ . NC-gd/gd had been judged by progeny testing. When a litter contained at least one F2 mouse with the mutant phenotype (gd/gd), both F1 dam and sire were judged to be gd/+ . When a litter contained only normal mice, all the F2 mice were discarded because either one or both of the F1 parents might have the +/+ genotype. Some of the F2 mice (n = 209) were harvested at the age of 25 days, and the remaining F2 mice (n = 160) were harvested on and after 30 days of age. When only the 25-day F2 mice were analyzed (n = 209), absolute body weight (ABS) was...
used as a trait value irrespective of sex. When all F2 mice were analyzed \((n = 369)\), the body weight of each individual was evaluated relative to that of the heaviest littermate (relative body weight [REL]) within each sex.

Quantitative trait locus (QTL) mapping analysis was performed with the MAPMAKER/EXP 3.0b and the MAPMAKER/QTL 1.1b computer programs (Lander et al. 1987). Although the mapping analysis was limited to chromosome 4, a genome-wide threshold significance at the \(\alpha = 0.05\) level, that is, logarithm of the odds (LOD) score \(\geq 4.3\) \((P = 5.2 \times 10^{-5})\), was adopted (Lander and Kruglyak 1995).

**Candidate Gene Analysis**

As described below, 2 similar mutant loci have been mapped to near the \(gd\) locus. One is Hertwig’s anemia \((an)\) (Hertwig 1942), and the other is reproductive mutant 10 \((repro10)\), JAX REPRODUCTIVE MUTAGENESIS program. In particular, Mouse Genome Informatics (MGI, operated by the Jackson Laboratory, Bar Harbor, ME) search offered 2 plausible candidate genes for \(repro10\), that is, \(ROD1\) regulator of differentiation 1 \((S.\) pombe \((Rod1)\) and hydroxysteroid dehydrogenase like 2 \((Hsdl2)\). Nucleotide sequences of open reading frame of these genes were determined in NC-\(gd/gd\) mice by use of cDNA derived from brain mRNA. DNA sequences were determined directly on the polymerase chain reaction (PCR) products by ABI PRISM Model 3100. The PCR primer sequences used were as follows: a primer set 5’-GGGGATCTGATGAGCTTCTG-3’ and 5’-GGGGATCTGATGAGCTTCTG-3’ for the first half of \(ROD1\) and 5’-TGAGCTGCTTCAGTCTGTGC-3’ for the second half of \(ROD1\), whereas 5’-CCACTGCCACCAAGCTCACCA-3’ and 5’-TGAGCTGCTTCAGTCTGTGC-3’ for the first half of \(Hsdl2\) and 5’-GCCAAAAGTTTTACTGTCAG-3’ for the second half of \(Hsdl2\). PCR was performed in 20 \(\mu l\) of reaction mixture containing 1\(\times\) PCR buffer (10 mM Tris–HCl, pH 8.3, and 50 mM KCl), 2.5 mM MgCl\(_2\), 320 \(\mu\)M deoxynucleoside triphosphate, and 1 U Taq DNA polymerase (Amplitaq Gold DNA polymerase, Applied Biosystems, Branchburg, NJ). Thermal conditions were as follows: initial denaturation for 12 min at 95 °C, followed by 35 cycles of 94 °C for 30 s, 60 °C for 60 s, and 72 °C for 90 s and 1 cycle of 7-min extension at 72 °C.

**Histology**

Pathohistologic comparisons of several parenchymatous organs, including lung, liver, spleen, kidney, testis, and ovary, were performed between NC-\(+/?\) and NC-\(gd/gd\). For this purpose, formalin-fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Results and Discussion**

**Onset and Features of \(gd\) Mutation**

Putative mutants \((gd/gd)\) were distinguishable from normal littermates \((+/?)\) at 2 or 3 weeks after birth, but depending on the circumstances, it usually took another few days to discriminate confidently between mutants and normal littermates (because the pup growth depends on litter size as well as the nursing ability of the mother). A typical \(gd/gd\) mouse is shown in Figure 1A with a \(+/?\) littermate. Both mice were 44-day-old males. The body weight of \(+/?\) was 25.54 g, whereas that of \(gd/gd\) was 8.44 g. Many \(gd/gd\) mice became wasted and/or haggard with advancing age, as seen in Figure 1B. The body weight of the mutants did not exceed 10 g throughout their life (Figure 2A).

**Histologic Comparison between \(gd/gd\) and \(+/?\)**

Histologic comparisons were made between \(gd/gd\) and \(+/?\) littermates (the testis was compared between inbred NC-strain littermates at the age of 41 days, whereas the ovary was compared between F2 littermates at the age of 5 weeks). Several parenchymatous organs, including histologic sections of lung, liver, kidney, spleen, testis, and ovary, were inspected. Overt histologic changes were detected only in the testis and ovary (Figures 3 and 4). In \(gd/gd\) testis, degeneration and/or necrosis were found in the seminiferous epithelium, particularly in the spermatocytes (Figure 3D,E);
Genetic Mapping of gd Locus on Chromosome 4

Of the total of 369 F2 mice, 83 (42 males and 41 females) were judged as being affected mutants (gd/gd), 275 as nonaffected normal (+/?, 139 males and 126 females), and the remaining 11 (5 males and 6 females) could not be classified (?/?, Table 1 and Figure 2B). In all, 196 were agouti (A/-; B/-), 74 were agouti-brown (A/-; b/b), 78 were black (a/a; B/-), and 21 were brown (a/a; b/b); AB:Ab/Ab:ab ratio was in good accordance with the expected Mendelian ratio of 9:3:3:1. However, when the gd/gd and +/? were analyzed separately, AB:Ab:Ab:ab did not follow the theoretical ratio. That is, 1) of 83 gd/gd, 9 were AB, 56 were Ab, 5 were ab, and 13 were ab2; 2) of 275 +/?, 185 were AB, 13 were Ab, 70 were ab2, and 7 were ab; and 3) of 11 ?, 2 were AB, 5 were Ab, 3 were ab, and 1 was ab. In both gd/gd and +/?; A/:-a/a ratio was approximately 3:1 (65:18 in gd/gd and 198:77 in +/?), whereas B/:-b/b ratio was clearly different from 3:1 (14:69 in gd/gd and 250:20 in +/?). These results suggested a linkage between gd locus and brown (b) locus (tyrosinase-related protein 1, Tyrp1) on chromosome 4. In addition, as the coat color ratio implied that there would be no prenatal lethality, gd, was confirmed to be inherited as a recessive trait.

The next step was to localize the gd locus to a region on chromosome 4. Because the gd locus is closely linked with the Tyrp1 locus on chromosome 4 and because the Tyrp1 locus is mapped to 38.0 cM on chromosome 4, I genotyped microsatellite markers that are polymorphic between NC and B6 in all F2 mice. However, because there are too few available microsatellite markers in a relevant region on chromosome 4, I adopted the QTL mapping strategy to localize the gd locus further. Because the most obvious mutant phenotype was growth retardation, body weight was the only reliable phenotypic variable in the following genetic mapping study. A plot of ABS is shown in Figure 2B. In Figure 2C, the ABS of F2 mice more than 25 days old is shown. The REL was calculated from these data. A QTL mapping analysis was performed on the 369 F2 mice. As expected, significant evidence of linkage was identified on chromosome 4, between microsatellite markers D4Mit139 and D4Mit178 (Figure 5). A significant QTL for ABS was identified at 30.6 cM, with a peak LOD score of 28.6, which explained 49.7% of the F2 variance (Table 2). A significant QTL for REL was also identified at 30.6 cM, with a peak LOD score 83.7, which explained 76.9% of the F2 variance. The large variance attributable to this locus further substantiated the monogenic nature of this mutation. The strength of the QTL allele effects on trait values was confirmed by 1-way analysis of variance by use of genotypes at the microsatellite markers that were localized at both sides of the QTL peak (Table 3). The results were coincident with the fact that the N allele at the gd locus is recessive to the B

![Figure 2](https://academic.oup.com/jhered/article-abstract/100/3/380/868033)

Figure 2.  Weight plot. (A) Comparative plots of body weight in NC-gd/gd and NC-+/? litters (♂: +/? males, ♀: +/? females, ♀: gd/gd males, ♀: gd/gd females). (B) Scatter plot of body weight in F2 mice at 25 days after birth. Each point represents ABS of an individual mouse. (C) Comparative plots of body weight in F2. Each point represents mean ± standard error of ABS of several mice (♂: +/? males, ♀: +/? females, ♀: gd/gd males, ♀: gd/gd females).

therefore, mutant males were thought to be sterile because of defective spermatogenesis. In gd/gd ovary, necrosis of granulosa cells and increased number of atretic follicles were remarkable (Figure 4D). As seen in Figure 4A, usually, more developing follicles can be observed in normal ovary. Mutant ovaries were generally atrophied (Figure 4B,C).
Figure 3. Histologic comparison of testis. (A) +/? (B–E) gd/gd. (A–C) Gross histology of testis. gd/gd testes show maturation arrest. These were photographed at the same magnification. (D, E) Degeneration and necrosis of spermatocytes are indicated by arrows, and vacuolar degeneration of them is indicated by an arrow head. Spermatogenesis is reduced.

Figure 4. Histologic comparison of ovary. (A) Gross histology of +/? ovary. Many developing follicles are observed. (B, C) Gross histology of gd/gd ovary. Mutant ovaries were atrophied. (D) An enlarged histologic figure of gd/gd ovary. Necrosis of granulosa cells (arrow heads) and increased number of atretic follicles (arrows) were remarkable.
allele and is associated with decreased body weight (Tables 2 and 3).

Further Mapping of gd Locus and Candidate Gene Sequencing

Because there were no available polymorphic microsatellite markers between D4Mit139 and D4Mit178, I further mapped the gd locus between these markers. Eighty-three F2 mice, which had been confidently judged as gd/gd, were again analyzed with the assumption that the gd locus genotype in these mice was homozygous for the N allele (N/N). As a result, the gd locus was suggested to be localized at the 30.5-cM position. This was very close to the position of the above-mentioned QTL peak. On the basis of the megabase pair position of D4Mit139 and D4Mit178, the physical position of the gd locus was estimated to be 58.3 Mbp. With the aid of MGI as a tool, genes that affect body size and/or male reproduction were searched for genomic region on chromosome between D4Mit139 (55.2 Mbp) and D4Mit178 (66.7 Mbp). Two plausible candidates were listed; one was an and the other was repro10 (http://reproductive-vegenomics.jax.org). The molecular nature of these mutations remains unknown. The an has been mapped to the 35.1-cM position. Although an/an mice are known to have a small body size, they usually have body weights greater than 10 g (Hertwig 1942). Thus, an may be excluded as a candidate for gd. On the other hand, the repro10 has been mapped to the 32.05-cM position. repro10/repro10 Mice were also shown to be small, and in particular, they resembled gd in that both have defects in the testis. Indeed, repro10/repro10 have defects in spermiogenesis (http://reproductive-vegenomics.jax.org/mutants/G1-422-35.html). MGI provided 2 candidate genes for repro10, that is, Rod1 and Hsd2. Rod1 is located at 59.5–59.6 Mbp, and Hsd2 is located at 59.6 Mbp on chromosome 4. Therefore, the nucleotide sequences of these genes were determined in a putative gd/gd mouse and compared with each reference sequence from the B6 strain. Consequently, no nucleotide alterations were identified in both genes between NC-gd/gd and B6, suggesting that these were clearly excluded from potential candidate genes. I have also done MGI search on the basis of gd/gd female phenotype. One candidate gene, Ambp gene exhibited abnormal ovarian folliculogenesis (Zhuo et al. 2001). However, Ambp is unlikely to be allelic with gd because mutant mice did not show growth defect and male mutants were fully fertile. In addition, as to the above-mentioned repro10, no apparent mutant phenotypes have been reported to date for repro10/repro10 females.

Table 1. Identification of linkage between gd locus and Typr1 locus on chromosome 4

| Coat color | Total no. of mice classified by coat color and gd locus genotype | AB | Ab | aB | ab |
|------------|-----------------------------------------------------------------|----|----|----|----|
| B6 × NC-gd/ + × F2 | 8 | 156 | 1 | 44 | 12 | 3 | 5 | 56 | 2 | 11 | 6 | 1 |
| NC-gd/ + × B6 F2 | 1 | 29 | 1 | 12 | 1 | 2 | 0 | 14 | 1 | 2 | 1 | 0 |
| Total no. (n = 369) | 9 | 185 | 2 | 56 | 13 | 5 | 5 | 70 | 3 | 13 | 7 | 1 |

* AB, agouti; Ab, agouti-brown; aB, black; ab, brown.

* gd/gd, F2 mice judged as affected mutant; +/?, F2 mice judged as nonaffected normal; ?/?, F2 mice that could not be classified.

Figure 5. LOD score plots for ABS and REL on chromosome 4.
Growth retardation mutations are frequently accompanied by defects in spermatogenesis (Cressman et al. 1999; Cha et al. 2004; Kress et al. 2007). For example, 

| Marker | Mean ± standard deviation | N/N | N/B | B/B | Nominal P value |
|--------|---------------------------|-----|-----|-----|-----------------|
| ABS D4Mit139 | 8.29 ± 3.22 (63) | 13.79 ± 3.03 (102) | 13.14 ± 2.78 (44) | 2.45 × 10⁻²³ |
| D4Mit178 | 7.92 ± 2.36 (58) | 13.88 ± 3.18 (102) | 12.90 ± 3.03 (49) | 3.31 × 10⁻²⁶ |
| REL D4Mit139 | 0.495 ± 0.204 (96) | 0.888 ± 0.120 (184) | 0.868 ± 0.127 (78) | 1.89 × 10⁻⁶⁶ |
| D4Mit178 | 0.491 ± 0.198 (91) | 0.887 ± 0.128 (183) | 0.852 ± 0.145 (84) | 1.74 × 10⁻⁶² |

Finally, the gd region of chromosome 4 roughly corresponds to human chromosome segment 9q32–q34, where no similar mutations had been reported. Although the molecular nature of this mutation is unclear, identification of gene that is responsible for gd mutation promises to make future contribution to relevant diseases in human beings.

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**References**

Cha KB, Karolyi IJ, Hunt A, Wenglikowski AM, Wilkinson JE, Dolan DF, Dootz G, Finnegan AA, Seasholtz AF, Hankenson KD, et al. 2004. Skeletal dysplasia and male infertility locus on mouse chromosome 9. Genomics. 83:951–960.

Cressman VL, Backlund DC, Averutskaya AV, Leadon SA, Godfrey V, Koller BH. 1999. Growth retardation, DNA repair defects, and lack of spermatogenesis in BRCA1-deficient mice. Mol Cell Biol. 19:7061–7075.

Hertwig P. 1942. Neue Mutationen und Koppelungsgruppen bei der Hausmaus. Z Indukt Abstamm Vererbungsl. 80:220–246.

Kress C, Gautier-Courteille C, Osborne HB, Babinet C, Paillard L. 2007. Inactivation of CUG-BP1/CELF1 causes growth, viability, and spermato-genesis defects in mice. Mol Cell Biol. 27:1146–1157.

Lander E, Kruglyak L. 1995. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet. 11:241–247.

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics. 1:174–181.

Zhuo L, Yoneda M, Zhao M, Yingyung W, Yoshida N, Kitagawa Y, Kawamura K, Suzuki T, Kimata K. 2001. Defect in SHAP-hyaluronan complex causes severe female infertility. A study by inactivation of the bikunin gene in mice. J Biol Chem. 276:7693–7696.

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