Further characterization of diabetes mellitus and body weight loss in males of the congenic mouse strain DDD.Cg-A\(^{f}\)

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ABSTRACT. The A\(^{f}\) allele at the agouti locus causes obesity and promotes linear growth in mice. However, body weight gain stops between 16 and 17 weeks after birth, and then, body weight decreases gradually in DDD.Cg-A\(^{f}\) male mice. Body weight loss is a consequence of diabetes mellitus, which is genetically controlled mainly by a quantitative trait locus (QTL) on chromosome 4. This study aimed to further characterize diabetes mellitus and body weight loss in DDD.Cg-A\(^{f}\) males. The number of \(\beta\)-cells was markedly reduced, and plasma insulin levels were very low in the DDD.Cg-A\(^{f}\) males. Using a backcross progeny of DDD \(\times (B6 \times DDD.Cg-A^{f})\) F\(_1\)-A\(^{f}\), we identified one significant QTL for plasma insulin levels on distal chromosome 4, which was coincidental with QTL for hyperglycemia and lower body weight. The DDD allele was associated with decreased plasma insulin levels. When the DDD.Cg-A\(^{f}\) males were housed under three different housing conditions [group housing (4 or 5 DDD.Cg-A\(^{f}\) and DDD males), individual housing (single DDD.Cg-A\(^{f}\) male) and single male housing with females (single DDD.Cg-A\(^{f}\) male with DDD.Cg-A\(^{f}\) or DDD females)], diabetes mellitus and body weight loss were most severely expressed in individually housed mice. Thus, the severity of diabetes and body weight loss in the DDD.Cg-A\(^{f}\) males was strongly influenced by the housing conditions. These results demonstrate that both genetic and nongenetic environmental factors are involved in the development of diabetes mellitus and body weight loss in the DDD.Cg-A\(^{f}\) males.

KEY WORDS: A\(^{f}\) allele, \(\beta\)-cell loss, DDD.Cg-A\(^{f}\) mouse, quantitative trait locus (QTL), reduced insulin level

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loss. For this purpose, we compared and analyzed growth curves and blood phenotypes among DDD.Cg-A\(^+\) males housed in one of the following conditions: (1) group housing (4 or 5 DDD.Cg-A\(^+\) males housed together), (2) individual housing (single DDD.Cg-A\(^+\) male) and (3) single male housing with females (single DDD.Cg-A\(^+\) male with DDD.Cg-A\(^+\) or DDD females).

**MATERIALS AND METHODS**

Mice: The inbred mouse DDD.Cg-A\(^+\), DDD, B6.Cg-A\(^-\) and B6 strains were maintained at the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). The DDD.Cg-A\(^+\) mouse strain was established by introgression of the A\(^+\) allele from the B6-A\(^+\) strain into the DDD strain by backcrossing for 12 generations [19]. Because the original DDD strain had an albino coat, congenic DDD.Cg-A\(^+\) mice were further intercrossed between yellow (A\(^+\)) and agouti (A\(^-\)) litters to eliminate the Tyr\(^+\) allele.

All mice were maintained in a specific pathogen-free facility with a regular light cycle and controlled temperature and humidity. Food (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and water were freely available throughout the experimental period. Unless specifically mentioned, 4–5 mice were group-housed during the experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee of NIAS.

**Blood phenotype analyses:** At 25 weeks of age, mice were euthanized with an overdose of ether after they were fasted for 4 hr. Whole blood was drawn from the heart into a plastic tube using heparin as an anticoagulant. Blood glucose levels were then determined using Glutest Pro R (Sanwa Kagaku Kenkusho Co., Ltd., Nagoya, Japan), according to the manufacturer’s instructions. Sample tubes were centrifuged at 7,000 rpm for 5 min at 4°C to separate plasma. The plasma samples were maintained at −70°C until use. Plasma glucose levels were determined using a clinical colorimetric kit (Glu- test Pro R, Sanwa Kagaku Kenkusho Co., Ltd., Nagoya, Japan), according to the procedure described previously [21]. In brief, mice were weaned at 4 weeks after birth. Body weights were measured weekly from 5 to 25 weeks of age and determined to the nearest 0.01 g using an electronic balance. The average body weights of mice from 5 to 25 weeks were monitored using the following equation:

\[
\text{Log (Body weight)} = a + b \times \text{Log (Week)} + c \times [\text{Log (Week)}]^2 + \epsilon
\]

The DDD.Cg-A\(^+\) males were housed under one of the following three different conditions: (1) group housing: 4 or 5 DDD.Cg-A\(^+\) males were housed together during the experimental period (DDD.Cg-A\(^+\) males housed under this condition are hereafter designated as GH); (2) individual housing: a single DDD.Cg-A\(^+\) male was individually housed during the experimental period (DDD.Cg-A\(^+\) males housed under this condition are hereafter designated as IH); and (3) single male housing with females: single DDD.Cg-A\(^+\) male was housed with 1–4 females (DDD.Cg-A\(^+\) or DDD) of the same litter during the experimental period (DDD.Cg-A\(^+\) males housed under this condition are hereafter designated as SF).

Growth curves of the DDD.Cg-A\(^+\) males were analyzed using the model defined by the above equation. Optimum estimates and 95% CI for the parameters were determined by the least-squares method weighted by the number of mice. Statistical significance was set at the a=0.05 level. Data analysis was performed using JMP ver. 9.0.1 statistical software (SAS Institute Inc., Cary, NC, U.S.A.).
RESULTS

Plasma insulin levels were reduced in DDD.Cg-Ay males: Table 1 shows the mean ± SE for plasma insulin levels in several inbred strains and their F1 mice at 25 weeks of age. The DDD.Cg-Ay males had substantially lower plasma insulin levels than other strains. The DDD.Cg-Ay males had significantly lower plasma insulin levels than the (B6 × DDD) F1-Ay males and B6.Cg-Ay males. Of note, the DDD.Cg-Ay males had lower plasma insulin levels than the non-Ay DDD males.

Decreased plasma insulin levels in DDD.Cg-Ay male were a consequence of pathological changes in islet β-cells: To determine whether the lower plasma insulin levels in the DDD.Cg-Ay males were due to pancreatic abnormalities, we performed histological analysis of the pancreas in one DDD male and three DDD.Cg-Ay males at 25 weeks of age. No notable histological changes were identified in the pancreas of the DDD male (Fig. 1A). In contrast, fibrosis of the pancreatic islets (Fig. 1B), vacuolar degeneration of the islet cells (Fig. 1C) and lymphocytic infiltration around the duct (Fig. 1D) were observed in the DDD.Cg-Ay males. We also noted that islets were reduced in the DDD.Cg-Ay males. Using anti-insulin antibody, we evaluated the quantity of insulin-releasing β-cells. In contrast to the result of immunohistochemical staining of normal islets in the DDD male (Fig. 2A), the number of β-cells was markedly reduced in the DDD.Cg-Ay males (Fig. 2B–2D). The number of the islets in a section and the average number of β-cells per islet correlated positively (r=0.6893) (Fig. 3).

Significant QTL for plasma insulin levels was identified on distal chromosome 4: To identify the gene or genes associated with lower plasma insulin levels in the DDD.Cg-Ay males, we performed QTL mapping in the BC progeny of crossed DDD males and DDD.Cg-Ay males (BC F1-Ay mice) and their F1 mice (BC F1-Ay mice). Plasma insulin levels in the BC F1-Ay mice did not follow a normal distribution (Fig. 4); therefore, QTL analysis was performed using a non-parametric procedure. We identified one significant QTL on distal chromosome 4 (Fig. 5A and 5B). The DDD allele was associated with decreased insulin levels at this locus [Mean ± SE insulin levels (ng/ml) in mice homozygous for the DDD allele (DDD/DDD) were 5.1 ± 0.6, and those in heterozygous (DDD/B6) mice were 12.9 ± 1.4].

Growth curves of DDD.Cg-Ay males differed among the three housing conditions: Growth analyses were performed on a separate mouse population from mice used for the above-mentioned experiments. Table 2 presents results of phenotypic measurements conducted according to housing conditions. In brief, experiments on individual housing were performed twice, i.e., the DDD.Cg-Ay males in the first, and second experiments were designated as IH_1 and IH_2, respectively. Growth curve analysis was performed only in the IH_1 mice, and plasma phenotype analyses were performed only in IH_2 mice.

DISCUSSION

Contrary to the expectation that Ay mice are invariably hyperinsulinemic, DDD.Cg-Ay males were hypoinsulinemic. In particular, plasma insulin levels were higher in the non-Ay DDD males than in the DDD.Cg-Ay males. We conclude that diabetes mellitus in the DDD.Cg-Ay males is a consequence of decreased insulin levels. Decreased insulin directly sug-
Fig. 1. Comparison of the histology of pancreatic islets (HE staining). (A) DDD male, (B)–(D) DDD Cg-Ay males. No notable abnormalities were found in the islets of the DDD male (A). In contrast, histological changes (surrounded by dotted lines), such as fibrosis (B), vacuolar degeneration (C) and lymphocytic infiltration around the ducts (D), were observed in the islets of the DDD Cg-Ay males. Scale bars=100 µm.

Fig. 2. Comparison of the histology of pancreatic islets (immunostaining). (A) DDD male, (B)–(D) DDD Cg-Ay males. The number of β-cells stained by anti-insulin antibody was markedly reduced in the islets of the DDD Cg-Ay males (B–D) compared with the DDD male (A). Scale bars=100 µm.
gests pathological changes in pancreatic islets. Although the size of the islets varied among the DDD.Cg- Ay males, the number of the islets was reduced in DDD.Cg- Ay males. All DDD.Cg- Ay mice showed islet fibrosis, which is observed in humans and various spontaneous rodent models of Type 2 diabetes mellitus [8]. Furthermore, all DDD.Cg- Ay males showed a loss of \( \beta \)-cells.

Because severe diabetes mellitus was not observed in the B6.Cg- Ay males, its development is specifically related to the DDD strain background. Thus, the DDD strain can be defined as having a diabetes-sensitive background, while the B6 strain has a diabetes-resistant background [21]. Such genetic background effect suggests the presence of modifier genes that functionally differ between the two strain backgrounds. QTL on chromosome 4, identified in the DDD.Cg-

![Fig. 3. Quantification of the islets and \( \beta \)-cells. Compared to DDD male, the number of the islets in a section and the average number of \( \beta \)-cells per islet were reduced in DDD.Cg- Ay males and correlated positively (r=0.6893).](image)

![Fig. 4. A histogram showing the distribution of plasma insulin levels in BC Ay mice. The approximate mean insulin values of the parental strains are indicated by arrows.](image)

![Fig. 5. QTL mapping of plasma insulin levels. (A) The genome-wide LOD score plot for plasma insulin levels in BC Ay mice. A horizontal dotted line indicates the genome-wide threshold LOD score for significant linkage. (B) Identification of a significant QTL on distal chromosome 4. Horizontal dotted lines indicate the threshold LOD scores for significant linkage (\( P<0.05 \), upper line) and suggestive linkage (\( P<0.63 \), lower line). A horizontal short line indicates 95% CI for the QTL.](image)

| Housing condition                              | n   | Analyses                                      |
|-----------------------------------------------|-----|-----------------------------------------------|
| Group housing (GH)                            | 14  | Growth curve ○                               |
| Individual housing (first, IH_1)              | 9   | Body weight at 25 weeks ○                    |
| Individual housing (second, IH_2)             | 16  | Blood glucose ○                              |
| Single male housing with females (SF)         | 8   | Plasma glucose ○                             |
|                                              |    | Plasma insulin ○                             |
|                                              |    | nd                                           |
|                                              |    | ○                                            |
| ○ indicates that the analysis was performed in the DDD.Cg- Ay males of the group, nd, not determined. |
$A'$ mice, was exactly the modifier locus that induced severe diabetes mellitus in the presence of the $A'$ allele. Similar results have been found for the obese gene mutations, $\text{Lep}^{ob}$ and $\text{Lepr}^{db}$ [5, 6, 9]. $\text{Lep}^{ob}$ or $\text{Lepr}^{db}$ mutations produce mild diabetes mellitus with hyperinsulinemia in the B6 background, whereas they produce severe diabetes mellitus with degenerative changes in the islets (including $\beta$-cell loss) in the C57BL/KsJ (KSJ) background. The B6 strain has been defined as the diabetes-resistant background, and the KSJ strain has been defined as the diabetes-sensitive background. These results suggest the presence of modifier genes that interact with $\text{Lep}^{ob}$ or $\text{Lepr}^{db}$ mutations; however, such modifier genes or loci have not yet been identified in the KSJ background. Although DDD.Cg-$A'$ and KSJ-$\text{Lepr}^{db}$/\text{Lepr}^{db} mice are similar in the point that both develop diabetes mellitus with $\beta$-cell loss, QTL on chromosome 4 is unlikely to serve as a modifier gene in the KSJ background because there are no conspicuous differences in the diabetes-related phenotypes induced by the $A'$ allele between the B6 and KSJ backgrounds [5].

Many BC $A'$ mice had higher plasma insulin levels than the parental strains (Fig. 3). We considered that such extreme values might be due to epistatic interactions between the loci. Although the plasma insulin levels did not follow a normal distribution, the results of QTL mapping performed using a parametric procedure were similar to those of QTL mapping performed using a non-parametric one (although the peak LOD score for QTL on chromosome 4 was changed); therefore, we performed pairwise scans. As a result, we did not find any potential interactions (data not shown).

Most importantly, QTL on chromosome 4 was coincident with that for body weight loss and blood/plasma glucose levels. The DDD allele was associated with decreased body weight, increased glucose levels and decreased insulin levels. For glucose levels, QTL on chromosome 4 was suggestive, and there were other suggestive QTLs on other chromosomes. In contrast, for insulin levels, the LOD score of QTL on chromosome 4 was very high, and there were no other QTLs. Taken together, the plasma insulin levels, therefore $\beta$-cell loss, were suggested to be controlled solely by the gene on chromosome 4. Inferring from the diabetes-related phenotypes in the KSJ-$\text{Lepr}^{db}$/\text{Lepr}^{db} mice, QTL on chromosome 4 will surely be related to the failure of $\beta$ cell expansion and islet atrophy [5, 6, 9].

In addition to the genetic aspect of diabetes mellitus, we need to pay attention to the non-genetic aspects of diabetes mellitus, because we obtained experimental results that the housing conditions affected the severity of diabetes mellitus.
and resulting body weight loss. The maximum body weight and the weeks at the maximum body weight clearly differed among the housing conditions.

As far as the blood/plasma glucose and plasma insulin levels were concerned, only IH mice were significantly different from the GH and SF mice, and no significant differences were observed between the GH and SF mice. This indicated that SF can be considered as a form of group housing. In other words, the significant difference between the IH and SF mice should not be attributed to the presence of females, but to the number of cohabiting mice. However, it was true that body weight loss was apparently ameliorated by the presence of the females.

Although the factors that link housing conditions and diabetes-related phenotypes were currently unknown, it was evident that the severity of diabetes and body weight loss in the DDD.Cg-A'y males was strongly influenced by the housing conditions. Similar to most spontaneous rodent models of Type 2 diabetes mellitus, diabetes mellitus, and therefore body weight loss, in the DDD.Cg-A'y males were also controlled by both genetic and non-genetic environmental factors.

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REFERENCES

1. Broman, K. W. and Sen, Ś. 2009. A guide to QTL mapping with R/qtl. Springer, New York.
2. Broman, K. W., Wu, H., Sen, S. and Churchill, G. A. 2003. R/qtl: QTL mapping in experimental crosses. Bioinformatics 19: 889–890. [Medline] [CrossRef]
3. Bultman, S. J., Michaud, E. J. and Woychik, R. P. 1992. Molecular characterization of the mouse agouti locus. Cell 71: 1195–1204. [Medline] [CrossRef]
4. Chen, A. S., Marsh, D. J., Trumbauer, M. E., Frazier, E. G., Guan, X. M., Yu, H., Rosenblum, C. I., Vongs, A., Feng, Y., Cao, L., Metzger, J. M., Strack, A. M., Camacho, R. E., Mellin, T. N., Nunes, C. N., Min, W., Fisher, J., Gopal-Truter, S., MacIntyre, D. E., Chen, H. Y. and Van der Ploeg, L. H. 2000. Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nat. Genet. 26: 97–102. [Medline] [CrossRef]
5. Coleman, D. L. 1978. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia 14: 141–148. [Medline] [CrossRef]
6. Coleman, D. L. and Hummel, K. P. 1973. The influence of genetic background on the expression of the obese (Ob) gene in the mouse. Diabetologia 9: 287–293. [Medline] [CrossRef]
7. Duhl, D. M., Vriel, H., Miller, K. A., Wolf, G. L. and Barsh, G. S. 1994. Neomorphic agouti mutations in obese yellow mice.
8. Homo-Delarche, F., Calderari, S., Irmlinger, J. C., Gangnerau, M. N., Coulaud, J., Rickenbach, K., Dolz, M., Halbin, P., Portha, B. and Serradas, P. 2006. Islet inflammation and fibrosis in a spontaneous model of type 2 diabetes, the GK rat. *Diabetes* **55**: 1625–1633. [Medline] [CrossRef]

9. Hummel, K. P., Coleman, D. L. and Lane, P. W. 1972. The influence of genetic background on expression of mutations at the diabetes locus in the mouse. I. C57BL-KsJ and C57BL-6J strains. *Biochim. Genet.* **7**: 1–13. [Medline] [CrossRef]

10. Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fang, Q., Berkemeier, L. R., Gu, W., Kesterson, R. A., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Burn, P. and Lee, F. 1997. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* **88**: 131–141. [Medline] [CrossRef]

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

12. Leibel, R. L., Chung, W. K. and Chua, S. C. Jr. 1997. The molecular genetics of rodent single gene obesities. *J. Biol. Chem.* **272**: 31937–31940. [Medline] [CrossRef]

13. Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overtorn, L., Kost, T., Luther, M., Chen, W., Woychik, R. P., Wilkison, W. O. and Cone, R. D. 1994. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* **371**: 799–802. [Medline] [CrossRef]

14. Michaud, E. J., Bultman, S. J., Stubbs, L. J. and Woychik, R. P. 1993. The embryonic lethality of homozygous lethal yellow mouse (*A/y*) is associated with the disruption of a novel RNA-binding protein. *Genes Dev.* **7** A: 1203–1213. [Medline] [CrossRef]

15. Michaud, E. J., Bultman, S. J., Klebig, M. L., van Vugt, M. J., Stubbs, L. J., Russell, L. B. and Woychik, R. P. 1994. A molecular model for the genetic and phenotypic characteristics of the mouse lethal yellow (*Ay*) mutation. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 2562–2566. [Medline] [CrossRef]

16. Miller, M. W., Duhl, D. M., Vrieling, H., Cordes, S. P., Ollmann, M. M., Winkes, B. M. and Barsh, G. S. 1993. Cloning of the mouse agouti gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. *Genes Dev.* **7**: 454–467. [Medline] [CrossRef]

17. Ollmann, M. M., Wilson, B. D., Yang, Y. K., Kerns, J. A., Chen, Y., Gantz, I. and Barsh, G. S. 1997. Antagonism of central melanocortin receptors *in vitro* and *in vivo* by agouti-related protein. *Science* **278**: 135–138. [Medline] [CrossRef]

18. Robbins, L. S., Nadeau, J. H., Johnson, K. R., Kelly, M. A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K. G. and Cone, R. D. 1993. Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell* **72**: 827–834. [Medline] [CrossRef]

19. Suto, J. 2009. The *Ay* allele at the agouti locus reduces the size and alters the shape of the mandible in mice. *Proc. Jpn. Acad., Ser. B, Phys. Biol. Sci.* **85**: 248–257. [Medline] [CrossRef]

20. Suto, J. 2011. Quantitative trait loci that control body weight and obesity in an F2 intercross between C57BL/6J and DDD.Cg-*Ay* mice. *J. Vet. Med. Sci.* **73**: 907–915. [Medline] [CrossRef]

21. Suto, J. and Satou, K. 2013. Genetic background (DDD/Sgn versus C57BL/6J) strongly influences postnatal growth of male mice carrying the *A(y)* allele at the agouti locus: identification of quantitative trait loci associated with diabetes and body weight loss. *BMC Genet.* **14**: 35. [Medline] [CrossRef]