FULL PAPER *Laboratory Animal Science*

Further characterization of diabetes mellitus and body weight loss in males of the congenic mouse strain DDD.Cg-A^y

Jun-ichi SUTO[1)]* and Kunio SATOU[2)]

[1)]*Agrogenomics Research Center, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8634, Japan*
[2)]*Center for Animal Disease Control and Prevention, National Institute of Animal Health, Tsukuba, Ibaraki 305-0856, Japan*

(Received 8 July 2014/Accepted 17 October 2014/Published online in J-STAGE 6 November 2014)

ABSTRACT. The A^y 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^y 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^y males. The number of β-cells was markedly reduced, and plasma insulin levels were very low in the DDD.Cg-A^y males. Using a backcross progeny of DDD × (B6 × DDD.Cg-A^y) F_1-A^y, 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^y males were housed under three different housing conditions [group housing (4 or 5 DDD.Cg-A^y and DDD males), individual housing (single DDD.Cg-A^y male) and single male housing with females (single DDD.Cg-A^y male with DDD.Cg-A^y 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^y 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^y males.

KEY WORDS: A^y allele, β-cell loss, DDD.Cg-A^y mouse, quantitative trait locus (QTL), reduced insulin level

doi: 10.1292/jvms.14-0351; *J. Vet. Med. Sci.* 77(2): 203–210, 2015

Cpe^{fat}, *Tub*^{tub}, *Lep*^{ob}, *Lepr*^{db} and A^y have been known as representative single gene obesity mutations in mice [12]. Among these, obesity in mice with A^y allele is moderate and occurs late compared with that in mice with the other four mutations. The A^y mice also show metabolic abnormalities, such as hyperglycemia and hyperinsulinemia, thus making them a suitable model system for the study of obesity-associated diabetes mellitus in humans.

In normal mice, the agouti gene is expressed only in the skin [3, 16], and it regulates pigmentation by serving as an inverse agonist of the melanocortin 1 receptor (MC1R) [13, 18]. However, in the A^y mice, the A^y allele is associated with a large deletion causes agouti gene expression to be aberrantly controlled by the unrelated *Raly* gene promoter, leading to its ectopic overexpression [7, 14–16]. As a result, the A^y mice have a yellow coat color and develop maturity-onset obesity. Obesity in A^y mice is considered a consequence of the agouti protein serving as a constitutive antagonist of MC3R and MC4R by mimicking the action of the agouti-related protein [4, 10, 17].

In addition to the two commercially available congenic mouse strains for the A^y allele, B6.Cg-A^y [C57BL/6J (B6) background] and KK.Cg-A^y [KK/Ta (KK) background], we have developed an additional congenic strain, DDD.Cg-A^y [DDD/Sgn (DDD) background] [19]. DDD.Cg-A^y females are characterized by their massive obesity compared with KK.Cg-A^y and B6.Cg-A^y females [20]. Mice carrying the A^y allele at the agouti locus become obese and are heavier than their non-A^y littermates with B6 and KK genetic backgrounds. However, in males with the DDD genetic background, e.g., DDD.Cg-A^y male mice, body weight gain stops between 16 and 17 weeks after birth, and then, body weight decreases gradually [21]. We showed that the body weight loss is a consequence of diabetes mellitus, which is controlled by a major quantitative trait locus (QTL) on chromosome 4. Thus, the DDD strain background has been suggested to be sensitive to obesity-associated diabetes mellitus. This was in contrast to the B6 strain background, which has been considered resistant to obesity-associated diabetes mellitus [5, 6, 9]. We have already shown that DDD.Cg-A^y males are hyperglycemic [21], but we do not know anything about the other aspects of diabetes mellitus. One of the aims of this study was to examine whether blood insulin levels and/or pancreatic islet histology are normal in DDD.Cg-A^y males.

During the course of the experiments of our previous study in which 4 or 5 DDD.Cg-A^y and DDD males were housed together [21], we noticed that the body weight of the DDD.Cg-A^y males never exceeded 50 g. However, we found obese DDD.Cg-A^y males weighing more than 50 g in breeding cages in which males and females were housed together. Therefore, we suspected that the growth of the DDD.Cg-A^y males was strongly influenced by housing conditions. Thus, another aim of this study was to determine whether housing conditions influenced diabetes mellitus and body weight

*CORRESPONDENCE TO: SUTO, J., Agrogenomics Research Center, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8634, Japan. e-mail: jsuto@affrc.go.jp

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

MATERIALS AND METHODS

Mice: The inbred mouse DDD.Cg-A^y, DDD, B6.Cg-A^y and B6 strains were maintained at the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). The DDD.Cg-A^y mouse strain was established by introgression of the A^y allele from the B6-A^y strain into the DDD strain by backcrossing for 12 generations [19]. Because the original DDD strain had an albino coat, congenic DDD.Cg-A^y mice were further intercrossed between yellow (A^y) and agouti (A) littermates to eliminate the Tyr^c 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 Kenkyusho 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 (Glucose C-II Test Wako; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma insulin levels were determined using a mouse insulin ELISA kit (AKRIN-011H; Shibayagi Co., Ltd., Shibukawa, Japan).

Histology of the pancreas: The pancreas of mice was removed at necropsy. Formalin-fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin. For the identification of the loss of islet β-cells, insulin immunostaining was performed using polyclonal guinea pig anti-swine insulin antibody [Dako ENVISION Kit/HRP (AEC), Dako Japan, Tokyo, Japan]. We evaluated the quantity of insulin-releasing β-cells. For this purpose, we randomly selected at most 5 islets, irrespective of the size, and determined the number of β-cells stained by anti-insulin antibody in each islet.

QTL mapping: QTL mapping analysis of the plasma insulin levels was performed in 100 A^y backcross (BC) progeny, as described previously [21]. In brief, we produced 196 BC males, comprising 100 A^y (BC A^y) and 96 non-A^y (BC non-A^y) males, from a genetic cross of ♀DDD × ♂ (♀B6 × ♂DDD.Cg-A^y) F_1-A^y mice. This backcrossing was chosen, because neither ♀B6 × ♂DDD.Cg-A^y F_1-A^y males nor ♀DDD × ♂B6.Cg-A^y F_1-A^y males showed body weight loss and the DDD alleles involved in this phenomenon were considered recessive to the B6 allele. Methods for genomic DNA isolation and genotyping of microsatellite markers essentially followed the procedures, described in our previous study [21]. We newly genotyped 18 additional microsatellite markers (*D3Mit346*, *D3Mit110*, *D3Mit323*, *D7Mit178*, *D7Mit57*, *D7Mit225*, *D7Mit229*, *D7Mit232*, *D7Mit317*, *D8Mit95*, *D8Mit272*, *D10Mit130*, *D10Mit180*, *D14Mit11*, *D14Mit107*, *D15Mit159*, *D19Mit16* and *D19Mit63*) in this study.

Of note, chromosome 7 is divided into two parts. Because of the introgression of the *Tyr* locus from the B6 strain, an intermediate part of the DDD genome on chromosome 7 is replaced by a B6 genome in the DDD.Cg-A^y mice. In this study, a region proximal to the B6 region was defined as chromosome 7.1 (*D7Mit178*, *D7Mit57*, *D7Mit225*, *D7Mit229*, *D7Mit232*, *D7Mit317* and *D7Mit250* were genotyped), whereas a region distal to the B6 region was defined as chromosome 7.2 (only *D7Mit362* was genotyped).

QTL mapping was performed using R/qtl [1, 2]. The threshold logarithm of odds (LOD) scores for suggestive (P<0.63) and significant (P<0.05) linkages was determined by performing 1,000 permutations for each trait [11]. For significant QTLs, the 95% confidence interval (CI) was defined by a decline of 1.5 LOD. After single QTL scans, pairwise evaluations for potential interactions between the loci were made. At this stage, the threshold LOD scores were strictly based on those recommended by Broman and Sen [1].

Body weight measurements and growth analysis: Body weight measurements and growth analysis were performed 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 modeled as the study outcomes using the following equation:

$$Log\ (Body\ weight) = a + b \times Log\ (Week) + c \times [Log\ (Week)]^2 + \mathcal{E}$$

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

Growth curves of the DDD.Cg-A^y 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 α=0.05 level. Data analysis was performed using JMP ver. 9.0.1 statistical software (SAS Institute Inc., Cary, NC, U.S.A.).

Statistics: For statistical comparisons between groups, the Tukey–Kramer honestly significant difference (HSD) test was used. *P* values of <0.05 were considered significant.

RESULTS

Plasma insulin levels were reduced in DDD.Cg-A^y males: Table 1 shows the mean ± SE for plasma insulin levels in several inbred strains and their F_1 mice at 25 weeks of age. The DDD.Cg-A^y males had substantially lower plasma insulin levels than other strains. The DDD.Cg-A^y males had significantly lower plasma insulin levels than the (B6 × DDD) F_1-A^y males and B6.Cg-A^y males. Of note, the DDD.Cg-A^y males had lower plasma insulin levels than the non-A^y DDD males.

Decreased plasma insulin levels in DDD.Cg-A^y male were a consequence of pathological changes in islet β-cells: To determine whether the lower plasma insulin levels in the DDD.Cg-A^y males were due to pancreatic abnormalities, we performed histological analysis of the pancreas in one DDD male and three DDD.Cg-A^y 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-A^y males. We also noted that the number of islets was reduced in the DDD.Cg-A^y 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-A^y 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-A^y males, we performed QTL mapping in the BC progeny of ♀DDD × ♂ (♀B6 × ♂DDD.Cg-A^y) F_1-A^y mice (BC A^y mice). Plasma insulin levels in the BC A^y 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 (*n*g/m*l*) 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-A^y 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-A^y 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.

Table 1. Plasma insulin levels in several inbred strains and their F_1 mice

Strain	n	Mean ± SE (*n*g/m*l*)
B6.Cg-A^y male	12	12.57 ± 3.18
DDD.Cg-A^y male	14	1.68 ± 0.28 [a, b]
DDD male	12	7.29 ± 1.14
(B6 × DDD.Cg-A^y) F_1-A^y male	12	23.22 ± 5.22
(DDD × B6.Cg-A^y) F_1-A^y male	9	13.11 ± 1.88
B6.Cg-A^y female	10	9.41 ± 1.34
DDD.Cg-A^y female	10	9.38 ± 2.13

a) Significant difference (P<0.0001) versus (B6 × DDD.Cg-A^y) F_1-A^y male, b) Significant difference (P<0.05) versus B6.Cg-A^y male.

We compared and analyzed the growth curves and blood phenotypes in the GH, IH and SF mice. Figure 6 shows a plot of the average body weight of the GH, IH (IH_1) and SF mice from 5 to 25 weeks of age. The average body weight of the IH_2 mice at 25 weeks is also presented. The maximum body weight of the GH mice was 43.5 g at 16.7 weeks (Table 3). The maximum body weight of the IH_1 mice was 42.9 g at 14.2 weeks, and that of the SF mice was 49.5 g at 19.3 weeks. Thus, the peak of the growth curve occurred in the order of the IH, GH and SF mice.

Individual housing significantly decreased and the presence of females significantly increased body weight at 25 weeks: In the IH_2 mice, body weight was determined only at 25 weeks. The average body weight of the IH_1 mice (40.06 g) was not significantly different from that of the IH_2 mice (37.94 g). Therefore, the data for the IH_1 and IH_2 mice were merged and analyzed (Fig. 7A). The IH mice were significantly lighter than the GH and SF mice. The SF mice were significantly heavier than the GH mice.

Individual housing substantially increased blood/plasma glucose levels and decreased plasma insulin levels: The average blood glucose level at 25 weeks in the IH_1 mice (537 mg/d*l*) was not significantly different from that in the IH_2 mice (592 mg/d*l*); therefore, the data for the IH_1 and IH_2 mice were merged and analyzed. Because 2 of the 9 IH_1 mice and 14 of the 16 IH_2 mice had blood glucose levels more than the upper limit of the measuring apparatus (600 mg/d*l*), their blood glucose levels were assumed to be 600 mg/d*l*. The IH mice had significantly higher blood and plasma glucose levels than the GH and SF mice, and no significant difference was observed between the GH and SF mice (Fig. 7B and 7C). Conversely, the IH mice had significantly lower plasma insulin levels than the GH and SF mice, and no significant difference was observed between the GH and SF mice (Fig. 7D).

DISCUSSION

Contrary to the expectation that A^y mice are invariably hyperinsulinemic, DDD.Cg-A^y males were hypoinsulinemic. In particular, plasma insulin levels were higher in the non-A^y DDD males than in the DDD.Cg-A^y males. We conclude that diabetes mellitus in the DDD.Cg-A^y males is a consequence of decreased insulin levels. Decreased insulin directly sug-

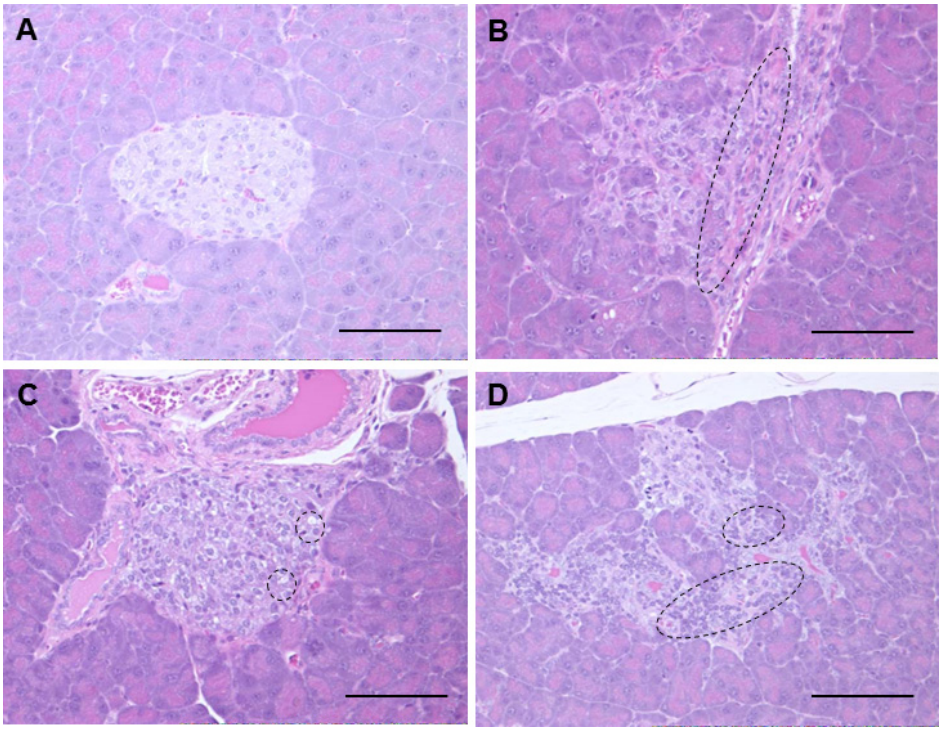

Fig. 1. Comparison of the histology of pancreatic islets (HE staining). (A) DDD male, (B)–(D) DDD.Cg-*A*^{y} males. No notable abnormalities were found in the islets of the DDD male (A). In contrast, histological changes (surrounded by dotted lines), such as fibrosis (B), vacuolar degeneration (C) and lymphocytic infiltration around the ducts (D), were observed in the islets of the DDD.Cg-*A*^{y} males. Scale bars=100 μm.

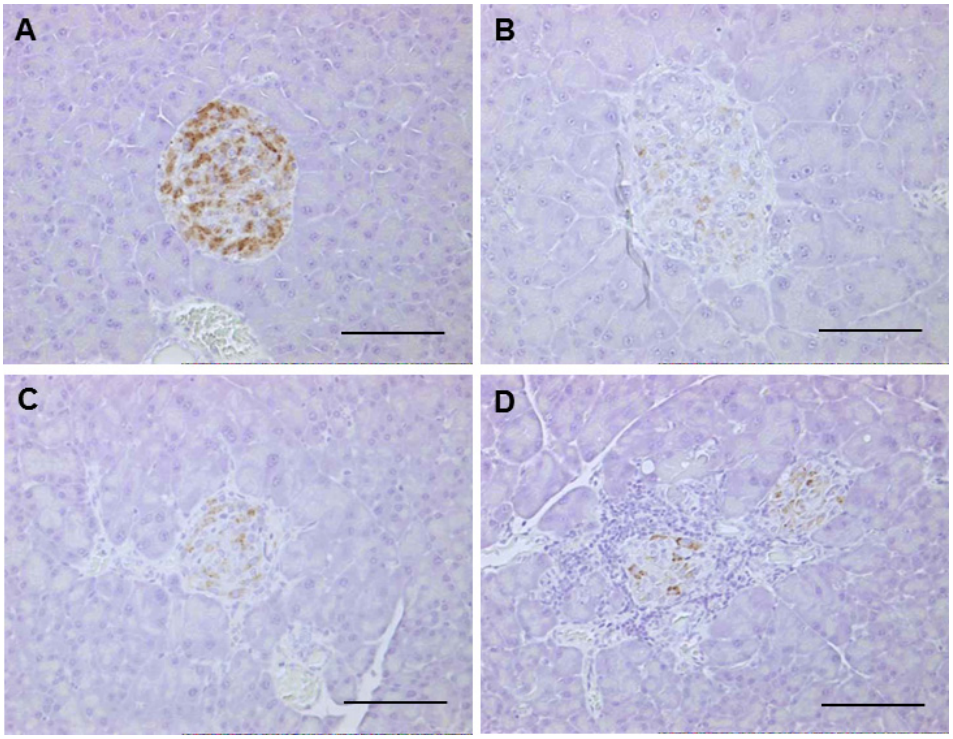

Fig. 2. Comparison of the histology of pancreatic islets (immunostaining). (A) DDD male, (B)–(D) DDD.Cg-*A*^{y} males. The number of β-cells stained by anti-insulin antibody was markedly reduced in the islets of the DDD.Cg-*A*^{y} males (B–D) compared with the DDD male (A). Scale bars=100 μm.

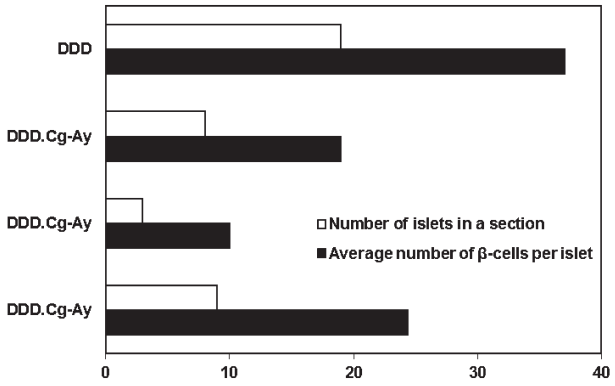

Fig. 3. Quantification of the islets and β-cells. Compared to DDD male, the number of the islets in a section and the average number of β-cells per islet were reduced in DDD.Cg-A^y males and correlated positively (r=0.6893).

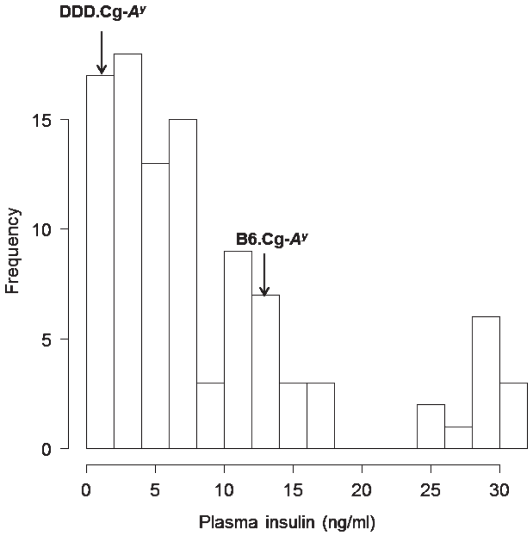

Fig. 4. A histogram showing the distribution of plasma insulin levels in BC A^y mice. The approximate mean insulin values of the parental strains are indicated by arrows.

gests pathological changes in pancreatic islets. Although the size of the islets varied among the DDD.Cg-A^y males, the number of the islets was reduced in DDD.Cg-A^y males. All DDD.Cg-A^y mice showed islet fibrosis, which is observed in humans and various spontaneous rodent models of Type 2 diabetes mellitus [8]. Furthermore, all DDD.Cg-A^y males showed a loss of β-cells.

Because severe diabetes mellitus was not observed in the B6.Cg-A^y 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-

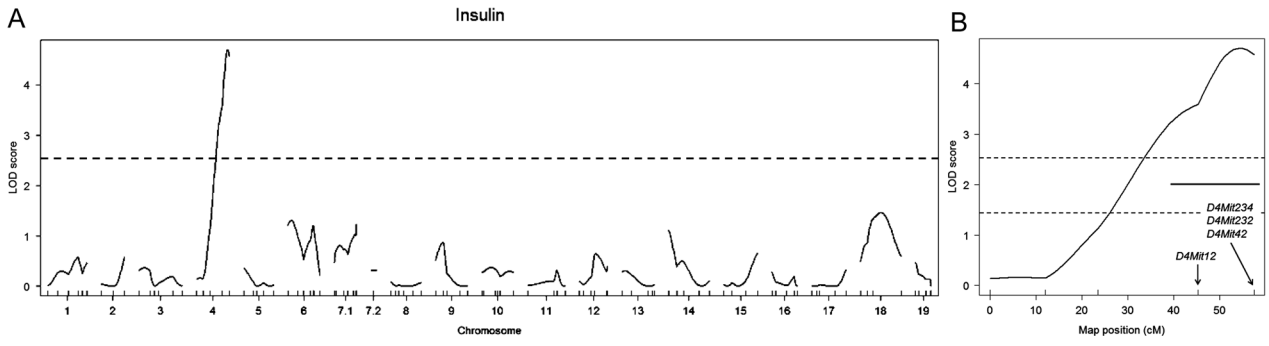

Fig. 5. QTL mapping of plasma insulin levels. (A) The genome-wide LOD score plot for plasma insulin levels in BC A^y mice. A horizontal dotted line indicates the genome-wide threshold LOD score for significant linkage. (B) Identification of a significant QTL on distal chromosome 4. Horizontal dotted 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.

Table 2. Phenotypic measurements conducted in terms of the housing conditions of DDD.Cg-A^y males

Housing condition	n	Analyses				
		Growth curve	Body weight at 25 weeks	Blood glucose	Plasma glucose	Plasma insulin
Group housing (GH)	14	○	○	○	○	○
Individual housing (first, IH_1)	9	○	○	○	nd	nd
Individual housing (second, IH_2)	16	nd	○	○	○	○
Single male housing with females (SF)	8	○	○	○	○	○

○ indicates that the analysis was performed in the DDD.Cg-A^y males of the group, nd, not determined.

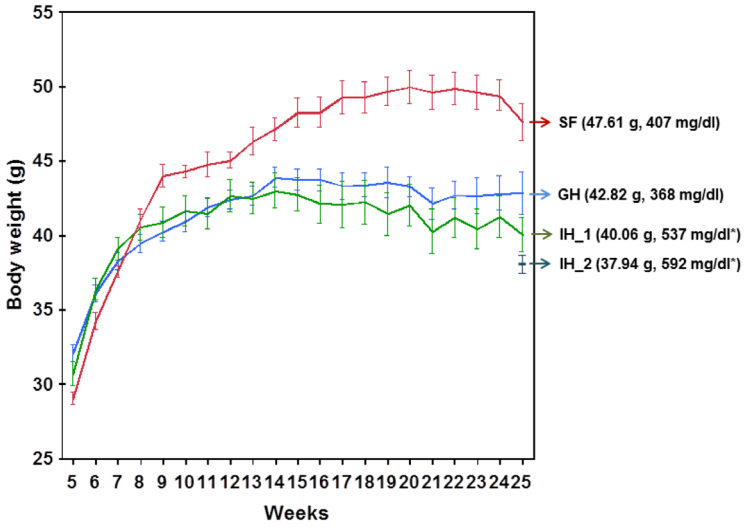

Fig. 6. Comparison of growth curves of DDD.Cg-A^y males subjected to the three different housing conditions. Body weight was determined from 5 to 25 weeks of age and was indicated by mean ± SE. Body weight and blood glucose levels at 25 weeks after 4 hr fasting are also presented.

Table 3. Parameters of growth curves

Housing condition	n	Parameter[a)]			Maximum body weight (g) (Weeks of age)
		Intercept	Log (Week)	(Log (Week))^2	
Group housing (GH)	14	2.226 ± 0.085	1.098 ± 0.071	−0.195 ± 0.014	43.5 (16.7)
Individual housing (IH_1)	9	1.927 ± 0.095	1.381 ± 0.078	−0.260 ± 0.016	42.9 (14.2)
Single male housing with females (SF)	8	1.493 ± 0.098	1.626 ± 0.081	−0.275 ± 0.016	49.5 (19.3)

a) Estimate ± SE.

A^y mice, was exactly the modifier locus that induced severe diabetes mellitus in the presence of the A^y allele.

Similar results have been found for the obese gene mutations, *Lep*^{ob} and *Lepr*^{db} [5, 6, 9]. *Lep*^{ob} or *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 β-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 *Lep*^{ob} or *Lepr*^{db} mutations; however, such modifier genes or loci have not yet been identified in the KSJ background. Although DDD.Cg-A^y and KSJ-*Lepr*^{db}/*Lepr*^{db} mice are similar in the point that both develop diabetes mellitus with β-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^y allele between the B6 and KSJ backgrounds [5].

Many BC A^y 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 β-cell loss, were suggested to be controlled solely by the gene on chromosome 4. Inferring from the diabetes-related phenotypes in the KSJ-*Lepr*^{db}/*Lepr*^{db} mice, QTL on chromosome 4 will surely be related to the failure of β 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

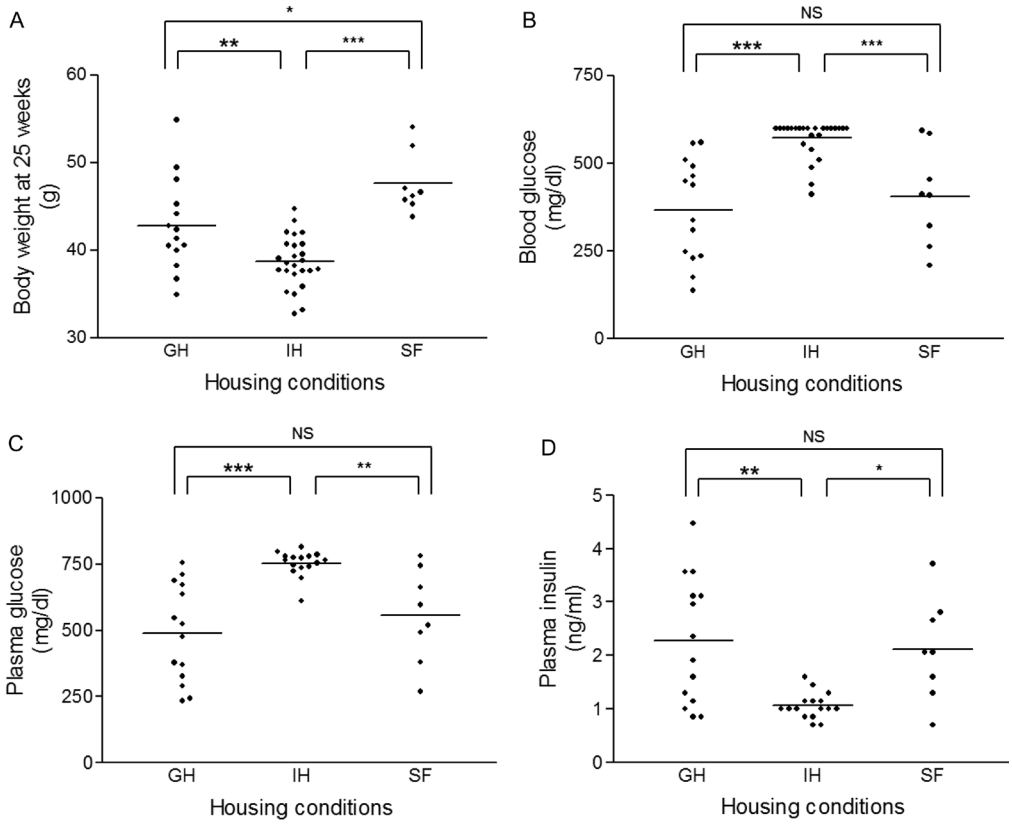

Fig. 7. Comparisons of body weights and blood phenotypes among DDD.Cg-A^y males subjected to the three different housing conditions. For body weights and blood glucose levels, the IH_1 and IH_2 mice were merged and analyzed as one group. Scatter plots of (A) body weight at 25 weeks, (B) blood glucose levels, (C) plasma glucose levels and (D) plasma insulin levels. Short horizontal lines indicate mean values.

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.

ACKNOWLEDGMENT. This study was supported in part by an institutional grant from NIAS.

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., Vrieling, H., Miller, K. A., Wolff, G. L. and Barsh, G. S. 1994. Neomorphic agouti mutations in obese yellow mice.

Nat. Genet. **8**: 59–65. [Medline] [CrossRef]
8. Homo-Delarche, F., Calderari, S., Irminger, J. C., Gangnerau, M. N., Coulaud, J., Rickenbach, K., Dolz, M., Halban, 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. *Biochem. 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., Overton, 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 mice (Ay/Ay) is associated with the disruption of a novel RNA-binding protein. *Genes Dev.* **7** 7A: 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 (A^y) 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 A^y 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 F_2 intercross between C57BL/6J and DDD.Cg-A^y 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]