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Diabetic modifier QTLs identified in F_2 intercrosses between Akita and A/J mice

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Abstract

To identify novel genetic modifiers of type 2 diabetes (T2D), we performed quantitative trait loci (QTL) analysis on F_2 progeny of hypoinsulinemic diabetic Akita mice, heterozygous for the *Ins2* gene Cys96Tyr mutation, and nondiabetic A/J mice. We generated 625 heterozygous (F_2-Hetero) and 338 wild-type (F_2-Wild) mice with regard to the *Ins2* mutation in F_2 intercross progeny. We measured quantitative traits, including plasma glucose and insulin concentrations during the intraperitoneal glucose tolerance test (IPGTT), and body weight (BW). We observed three significant QTLs in hypoinsulinemic hyperglycemic male F_2-Hetero mice, designated *Dbm1*, *Dbm3*, and *Dbm4* on Chromosomes 6, 14, and 15, respectively. They showed linkage to plasma glucose concentrations, with significant maximum logarithm of odds (LOD) scores of 4.12, 4.17, and 6.17, respectively, all exceeding threshold values by permutation tests. In normoinsulinemic normoglycemic male F_2-Wild mice, *Dbm1* on Chromosome 6 showed linkage to both plasma insulin concentrations and BW, and *Dbm2* on Chromosome 11 showed linkage to plasma glucose concentrations only, with LOD scores of 4.52 and 6.32, and 5.78, respectively. Based on these results, we concluded that *Dbm1*, *Dbm2*, *Dbm3*, and *Dbm4* represent four major modifier QTLs specifically affecting T2D-related traits and that these diabetic modifier QTLs are conditional on the heterozygous *Ins2* gene mutation and sex to exert their modifier functions. Identification of the genes responsible for these QTLs would provide new drug development targets for human T2D.

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Introduction

Type 2 diabetes (T2D) is a multifactorial disorder caused by both genetic (Leiter 1989, 1993) and environmental factors. Genetic factors can affect both insulin sensitivity/resistance and insulin secretion from pancreatic islet β cells (DeFronzo 1988). Although some genetic factors for maturity-onset diabetes in the young (MODY) have been identified in humans (Froguel et al. 1997), the multiple genetic factors that modify T2D-related traits remain to be identified. Quantitative trait loci (QTL) analysis has been applied to several rodent models of T2D to identify chromosomal loci for genetic modifiers. QTL analysis of obese animal models of T2D such as the OLETF rat (Kim et al. 1998), the KKA^y mouse (Suto et al. 1998a, b), the NZO mouse (Reifsnyder et al. 2000; Taylor et al. 2001), and the TSOD mouse (Hirayama et al. 1999), as well as nonobese animal T2D models such as the GK rat (Galli et al. 1996; Gauguier et al. 1996) and the SDT rat (Masuyama et al. 2003), have identified QTLs that influence factors such as plasma glucose and insulin concentrations, body weight (BW), and fat weight. While a number of genes have been implicated in monogenic etiologies for T2D from animal models of diabetes, including *ob*, *db*, *Tub*, A^y, and *Agrp*, none of these genes appears to explain the genetic background of most T2D cases in humans. To identify genetic modifiers of T2D that affect insulin sensitivity or resistance in the absence of obesity, we performed QTL analysis on F_2 intercross progeny of nonobese diabetic Akita and nondiabetic A/J mice.

The Akita mice were established as a nonobese T2D mouse model on the C57BL/6 (B6) genetic background (Yoshioka et al. 1997). A heterozygous mutation of the insulin 2 gene (*Ins2*; Cys96Tyr) has been identified as the etiology for the hypoinsulinemic diabetes in this mouse strain (Yoshioka et al. 1997; Kayo and Koizumi 1998; Wang et al. 1999). This mutation disrupts an intramolecular disulfide bond between the A and B chains of insulin generated from the insulin 2 gene. The misfolded insulin then causes endoplasmic reticulum (ER) stress, inducing expression of the ER stress-associated apoptosis factor C/EBP homologous protein (Chop, also known as GADD153) in pancreatic islets (Ron 2002; Oyadomari et al. 2002). Because disruption of the *Chop* gene in Akita mice delays the onset of diabetes, it appears that apoptosis of pancreatic islet β cells in Akita mice is induced via Chop (Oyadomari et al. 2002). Also, intracellular accumulation of misfolded proinsulin 2 has been reported to cause organelle dysfunction in pancreatic islet β cells and to suppress the secretion of coexisting wild-type insulin (Izumi et al. 2003). Thus, the Akita mouse model of nonobese T2D contains a defect in the glucose-responsive insulin secretion system that results in hypoinsulinemia.

To our knowledge, this is the first QTL analysis performed on a mouse model of insulin-deficient nonobese T2D, and it takes advantage of the genetically determined molecular defect present in the Akita mouse. In this study we report novel diabetic modifier QTLs detected in F_2 intercross mice derived from nonobese diabetic Akita and nondiabetic A/J mice strains.

Materials and methods

Mouse strains and creation of F_2 intercross mice. Male Akita mice and female A/J mice at 7 weeks of age were purchased from SLC Japan (Shizuoka, Japan). Mice were maintained with free access to food (CRF-1, purchased from Oriental Yeast) and water in a temperature- and humidity-controlled environment under a 12 h light–dark cycle. Akita mice were crossed with the A/J mice to produce F_1 progeny heterozygous for the mutation at the *Ins2* locus carried by Akita mice. F_1 heterozygous mice were then intercrossed to obtain F_2 mice. Of the 1305 F_2 mice produced, 682 were male and 623 were female. Of the male and female mice, 187 and 155, respectively, were homozygous for Cys96Tyr mutation at the *Ins2* locus (F_2-Homo); 171 and 167, respectively, were homozygous for the wild-type *Ins2* locus (F_2-Wild); and 324 and 301, respectively, were heterozygous (F_2-Hetero). Because most of the F_2-Homo mice (299 of 342) died from hyperglycemia before 10 weeks of age, F_2-Homo phenotypes were not monitored.

Genetic diagnosis of the insulin 2 gene mutation in F_1 and F_2 mice. Genomic DNA was extracted from the tail of each mouse at 4 weeks of age using the automatic DNA Isolation System NA-1000 (Kurabo, Osaka, Japan). *Ins2* genotype was determined by restriction fragment length polymorphism (RFLP) analysis as previously described (Wang et al. 1999). Briefly, polymerase chain reaction (PCR) amplification was carried out using 10 pmol sense and antisense primers, 200 μM dNTP mix, 25–50 ng genomic DNA, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) in a total volume of 50 μl. PCR conditions used were an initial denaturation at 94°C for 10 min, then 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 1 min, with a final extension at 72°C for 10 min. The primers (sense: 5′-TGCTGA TGCCCTGGCCTGCT-3′ and antisense: 5′-TGGT CCCACATATGCACATG-3′) produced a 280-bp PCR amplicon. PCR products were digested with the restriction enzyme *Ita*I (Roche Diagnostics GmbH, Penzberg, Germany) and separated by electrophoresis. The *Ins2* missense mutation of the Akita mouse disrupts an *Ita*I site, such that the expected sizes of the restriction fragments were 139 and 141 bp in F_1- or F_2-Wild mice, 139, 141, and 280 bp in F_1- or F_2-Hetero mice, and 280 bp in F_2-Homo mice.

Phenotyping of F_2 mice. Each F_2 mouse pup was weaned at 4 weeks of age. At 10 weeks of age, individual BW of F_2-Hetero and F_2-Wild mice were measured after overnight fasting for 16 h. The intraperitoneal glucose tolerance test (IPGTT) was performed on 10-week-old mice. After fasting for 16 h, 2 mg/g BW of glucose in physiologic saline was administered intraperitoneally, and blood was sampled from the retro-orbital sinus using a capillary pipette before (designated as 0 min) and 30, 60, and 120 min after glucose administration. Plasma samples were obtained by centrifugation, and plasma glucose concentrations were measured by the mutarotase-glucose oxidase method using the Glucose CII Test Wako (Wako, Osaka, Japan). Plasma immunoreactive insulin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using the Insulin ELISA Kit (Morinaga Institute of Biological Science, Yokohama, Japan).

Genome-wide genotyping of F_2 mice. Primers for microsatellite markers were designed using DNA sequences obtained from Mouse Genome Infomatics (MGI) of The Jackson Laboratory (http://www.informatics.jax.org) or the Whitehead Institute/MIT Center for Genome Research (http://www-genome.wi.mit.edu/cgi-bin/mouse/index). All primers were purchased from Applied Biosystems. The sense primer of each primer pair was labeled at the 5′ end with FAM, VIC, NED, or PET fluorescent dye. A total of 281 well-amplified microsatellite markers polymorphic between Akita and A/J mice with differences of 2 bp or more were selected. Sequences of primers are available on request. We genotyped all markers by PCR using unilaterally labeled fluorescent primers, dNTP mix, genomic DNA, and Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). PCR was performed according to the manufacturer's instructions, and amplicons were electrophoretically separated on a 3700 capillary DNA sequencer (Applied Biosystems). Analysis and positional assignment of each marker was performed using GeneScan v3.7 (Applied Biosystems) and Genotyper v3.7 (Applied Biosystems). We constructed linkage maps for all chromosomes, performed bootstrap QTL analysis and permutation tests, and examined pairwise interaction of QTLs with MapManager QTXb17 (Manly and Olson 1999). We calculated threshold logarithm of odds (LOD) values for significant or suggestive linkage based on permutation tests. We applied trait-specific significance threshold values obtained by permutation tests for multiple testing, with LOD scores of 2.8 and 4.3 set as limits for suggestive and significant levels, respectively (Lander and Kruglyak 1995). We performed the covariate-dependent single-locus genome scan using the ''scanone'' function of R/qtl software (http://www.biostat.jhsph.edu/~kbroman/qtl/) as described previously (Solberg et al. 2004).

Statistical analysis. Data were presented as mean ± standard deviation (SD) or ± standard error of the mean (SEM). Statistical analysis of measured traits was carried out using GraphPad InStat v3.05 (GraphPad Software Inc., San Diego, CA). Differences in standard deviations between genotype groups were tested using the *F* test or Bartlett's test. Phenotype comparisons between different genotype groups were performed using the Kruskal–Wallis test with Dunnett's multiple comparison post test.

Results

Phenotypic characterization of F_2 intercross mice. Table 1 and Supplementary Fig. 1 show, respectively, a summary and frequency distribution of the diabetes- and obesity-related phenotypes as determined by IPGTT on Akita, A/J, B6, and F_2 mice (F_2-Hetero, F_2-Wild). Plasma glucose concentrations were higher and insulin concentrations and BW were lower in Akita mice compared to nondiabetic A/J and B6 mice. Diabetic symptoms in Akita and F_2-Hetero mice were more severe in male mice than those in female mice, with higher fasting plasma glucose concentrations and decreased glucose clearance after glucose administration. These sex-dependent differences in IPGTT results were significant at 0, 30, 60, and 120 min ($p < 0.001$ at 0 min and $p < 0.01$ at 120 min for Akita mice; $p < 0.0001$ at each time point for F_2-Hetero mice), so that male and female Akita mice may act as models of severe and mild diabetes, respectively. Male F_2-Wild mice also showed increased glucose concentrations compared to female F_2-Wild mice, with significant differences in IPGTT results observed at 0, 30, 60, and 120 min ($p < 0.01$ at 0 min and $p < 0.0001$ at other time points).

For the F_2 intercross mice, both male and female F_2-Hetero mice displayed stronger diabetic phenotypes than F_2-Wild mice, with significant differences in IPGTT results at 0, 30, 60, and 120 min ($p < 0.0001$ at each time point). B6 mice tended to exhibit higher plasma glucose concentrations than A/J mice ($p < 0.05$ at 0 min and $p < 0.01$ at 120 min in males; $p < 0.01$ at 120 min in females), as well as higher insulin concentrations ($p < 0.05$ at 30 min in males; $p < 0.05$ at 120 min in females).

The standard deviations of plasma glucose and insulin concentrations at 0, 30, 60, and 120 min, as measured during IPGTT, and BW in male and female F_2-Hetero mice were higher than those observed in the respective parental male and female inbred Akita mice. Likewise, the standard deviations of plasma glucose and insulin concentrations in male and female F_2-Wild mice were larger than those observed in the respective parental male and female inbred A/J or B6 mice (Table 1, Supplementary Fig. 1).

Plasma glucose concentrations at 0 min in male and female F_2-Hetero mice (280.5 and 117.5 mg/dl, respectively) were lower than those in parental Akita mice (339.4 or 261.5 mg/dl, respectively) ($p < 0.01$ and $p < 0.0001$, respectively). BWs of male and female F_2-Hetero mice (20.5 and 19.0 g, respectively) were significantly heavier than those of Akita mice (17.4 or 16.9 g, respectively) ($p < 0.0001$ for both). Plasma insulin concentrations at 120 min in male and female F_2-Hetero mice (145.2 and 326.3 pg/ml, respectively) were also higher than thoses in Akita mice (53.1 and 97.7 pg/ml, respectively) ($p < 0.01$ and $p < 0.0001$, respectively).

Table 1. Phenotypic characteristics of parental inbred strains and F_2 intercross mice

Sex	*Male*				
Strain	*Akita*	*B6*	*A/J*	*F_2-Hetero*	*F_2-Wild*
Number of mice	*7*	*10*	*6*	*319*	*170*
Plasma Glucose Conc. (mg/dL)					
ipGTT 0 min	339.4 ± 30.1	92.2 ± 15.7	74.9 ± 10.2	280.5 ± 123.7	79.3 ± 19.3
ipGTT 30 min	630.7 ± 122.0	467.1 ± 91.0	401.0 ± 71.6	703.5 ± 138.1	448.9 ± 87.3
ipGTT 60 min	551.8 ± 90.4	338.8 ± 83.0	279.0 ± 100.5	668.4 ± 141.3	374.7 ± 103.6
ipGTT 120 min	521.4 ± 109.6	192.4 ± 45.6	120.6 ± 32.1	566.8 ± 149.3	255.7 ± 114.1
Plasma Insulin Conc. (pg/mL)					
ipGTT 0 min	45.9 ± 42.9	167.6 ± 243.1	198.1 ± 124.2	94.7 ± 102.7	200.3 ± 159.1
ipGTT 30 min	54.7 ± 62.7	555.2 ± 208.6	374.0 ± 49.7	77.8 ± 84.4	610.6 ± 284.3
ipGTT 60 min	47.8 ± 64.4	553.5 ± 339.0	358.1 ± 100.3	85.5 ± 80.6	585.5 ± 275.2
ipGTT 120 min	53.1 ± 53.7	530.8 ± 182.4	423.0 ± 94.3	145.2 ± 114.4	845.1 ± 372.8
Body Weight (g)	17.4 ± 1.3	22.0 ± 1.6	21.8 ± 1.6	20.5 ± 1.9	23.8 ± 2.9
Sex	*Female*				
Strain	*Akita*	*B6*	*A/J*	*F_2-Hetero*	*F_2-Wild*
Number of mice	*8*	*9*	*7*	*298*	*166*
Plasma Glucose Conc. (mg/dL)					
ipGTT 0 min	261.5 ± 37.0	100.4 ± 16.1	91.3 ± 17.5	117.5 ± 45.0	73.0 ± 18.5
ipGTT 30 min	600.8 ± 70.8	392.2 ± 72.8	324.5 ± 73.4	551.4 ± 103.6	353.7 ± 106.2
ipGTT 60 min	482.3 ± 58.8	232.2 ± 29.0	189.2 ± 59.5	486.2 ± 96.7	233.9 ± 95.0
ipGTT 120 min	376.6 ± 61.0	140.9 ± 28.9	99.3 ± 12.7	326.0 ± 106.3	134.6 ± 69.3
Plasma Insulin Conc. (pg/mL)					
ipGTT 0 min	31.6 ± 34.2	176.3 ± 144.1	63.1 ± 43.8	116.8 ± 117.4	150.7 ± 170.1
ipGTT 30 min	44.3 ± 47.2	554.7 ± 196.6	439.2 ± 135.6	105.2 ± 84.5	590.4 ± 264.3
ipGTT 60 min	86.5 ± 48.5	444.6 ± 135.3	330.3 ± 142.2	159.0 ± 105.6	546.3 ± 281.7
ipGTT 120 min	97.7 ± 69.3	406.3 ± 144.3	234.8 ± 143.4	326.3 ± 173.3	535.3 ± 348.1
Body Weight (g)	16.9 ± 0.4	17.6 ± 0.8	18.2 ± 0.7	19.0 ± 1.9	19.5 ± 2.3

All phenotypes were determined at 10 weeks of age in each group. Data are shown as means ± S.D.

Plasma glucose and insulin concentrations at 120 min in male F_2-Wild mice (255.7 mg/dl and 845.1 pg/ml, respectively) were higher than those in male A/J (120.6 mg/dl and 423.0 pg/ml, respectively) ($p < 0.0001$ for both) and B6 (192.4 mg/dl and 530.8 pg/ml, respectively) mice ($p < 0.01$ and $p < 0.001$, respectively). Plasma insulin concentrations at 120 min in female F_2-Wild mice (535.3 pg/ml) were higher than those in A/J (234.8 pg/ml) ($p < 0.001$) and B6 mice (406.3 pg/ml) ($p < 0.05$). BWs of male and female F_2-Wild mice (23.8 and 19.5 g, respectively) were greater than those in A/J (21.8 and 18.2 g, respectively) ($p < 0.05$ and $p < 0.01$, respectively) and B6 mice (22.0 and 17.6 g, respectively) ($p < 0.01$ and $p < 0.0001$, respectively).

Dbm1 on Chromosome 6. Because the diabetes-related traits in the presence or absence of the *Ins2* mutation were significantly different between F_2-Hetero and F_2-Wild mice, we performed separate genome-wide scans on F_2-Hetero and F_2-Wild mice to identify modifier QTLs that affected these diabetes-related traits, including plasma glucose and insulin concentrations during IPGTT and BW. We also analyzed male and female mice separately because of the sex-dependent differences in diabetes-related phenotypes.

The first QTL identified in male F_2-Hetero mice was located on Chromosome 6 and showed linkage to plasma glucose concentrations at 0 and 120 min during IPGTT (Fig. 1). The region near the *D6Mit31* locus had a maximum LOD score of 4.12 for fasting plasma glucose concentrations, with a second suggestive linkage observed close to the markers *D6Mit286* and *D6Nds5* with a LOD score of 3.47 and a third suggestive linkage observed near *D6Mit84* with a LOD score of 3.00 (Fig. 1a, c). Bootstrap analysis supported the LOD score peak at *D6Mit31*. We also found overlapping LOD score peaks near *D6Nds5* that affected plasma glucose concentrations at 120 min with a LOD score of 3.91, and a second suggestive linkage near *D6Mit84* showing a LOD score of 3.80 (Fig. 1b, c). Bootstrap analysis supported the LOD score peaks at both *D6Nds5* and *D6Mit84*. LOD scores at *D6Nds5* for plasma glucose concentrations at 30 and 60 min during IPGTT were 2.02 and 2.48, respectively. According to permutation tests, threshold LOD

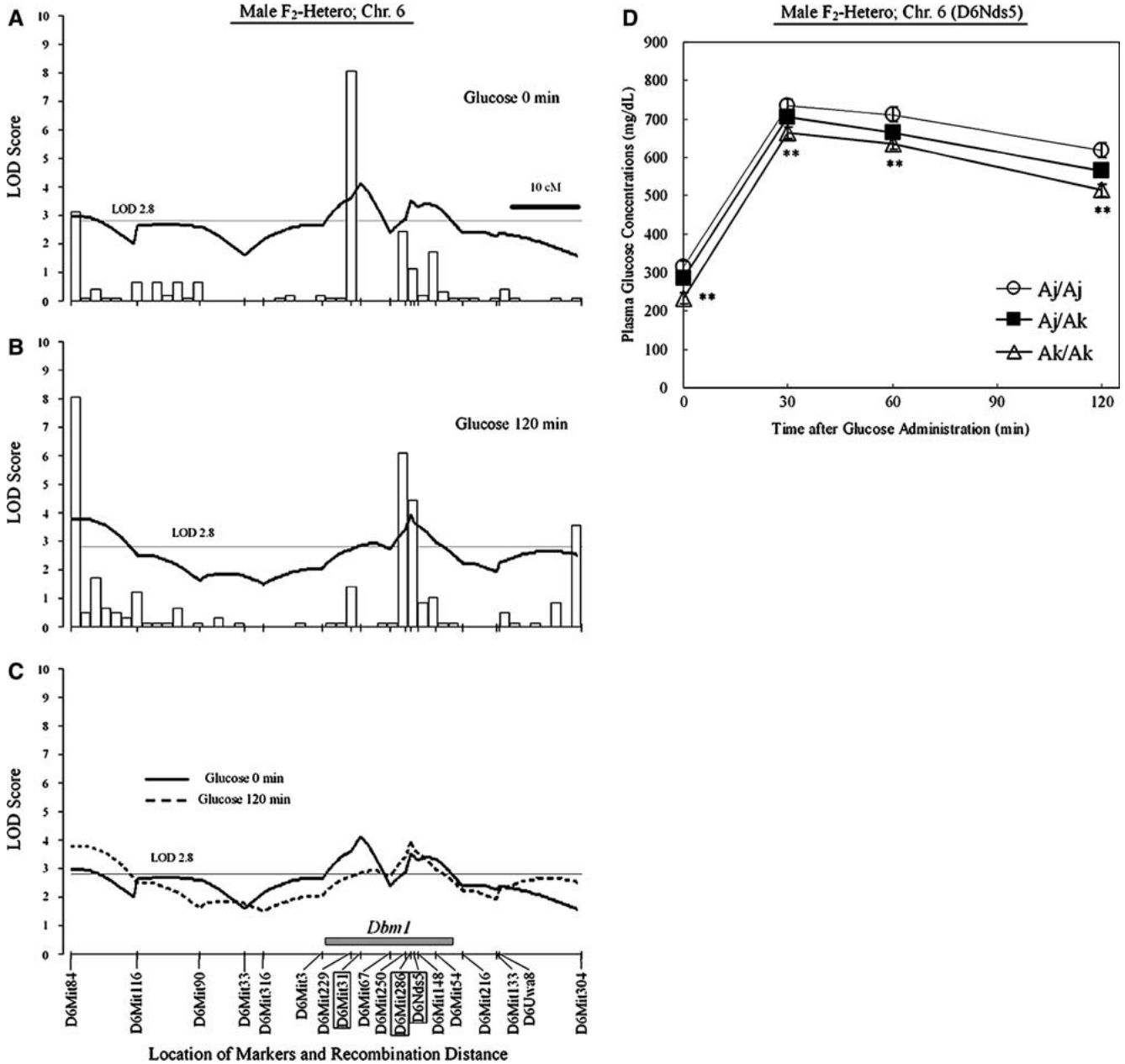


Fig. 1. LOD score plots of overlapping QTL on Chromosome 6 (*Dbm1*) and plasma glucose concentrations during IPGTT according to *D6Nds5* genotype in male F_2-Hetero mice. (**a**–**c**) The horizontal line indicates the threshold LOD score value of 2.8 for a suggestive linkage. The short bold line shows a genetic distance of 10 cM. The location of markers showing the maximum LOD scores in this analysis, *D6Mit31*, *D6Mit286*, and *D6Nds5*, are boxed. LOD score plots and bootstrap analysis are shown for plasma glucose concentrations at (**a**) 0 and (**b**) 120 min during IPGTT. (**c**) LOD score plots for plasma glucose concentrations at 0 (solid line) and 120 min (broken line). (**d**) Plasma glucose concentrations during IPGTT after 16 h fasting. Open circles denote mice homozygous for the A/J allele (Aj/Aj, n = 70). Filled squares denote heterozygous mice (Aj/Ak, n = 184). Open triangles denote mice homozygous for the Akita allele (Ak/Ak, n = 64). Data shown as mean ± SEM. Statistical significance was shown for $p < 0.05$ * and $p < 0.01$ ** between the Ak/Ak and Aj/Aj genotypes.

values for a significant QTL in male F_2-Hetero mice were 3.63, 3.60, 3.60, and 3.60 for plasma glucose concentrations at 0, 30, 60, and 120 min, respectively, based on 1000 permutations. The permutation thresholds for a suggestive linkage for these traits were 2.24, 2.24, 2.28, and 2.24, respectively, in male F_2-Hetero mice.

The strain-dependent effect of this QTL on plasma glucose concentrations in male F_2-Hetero mice based on genotype at *D6Nds5* is shown in

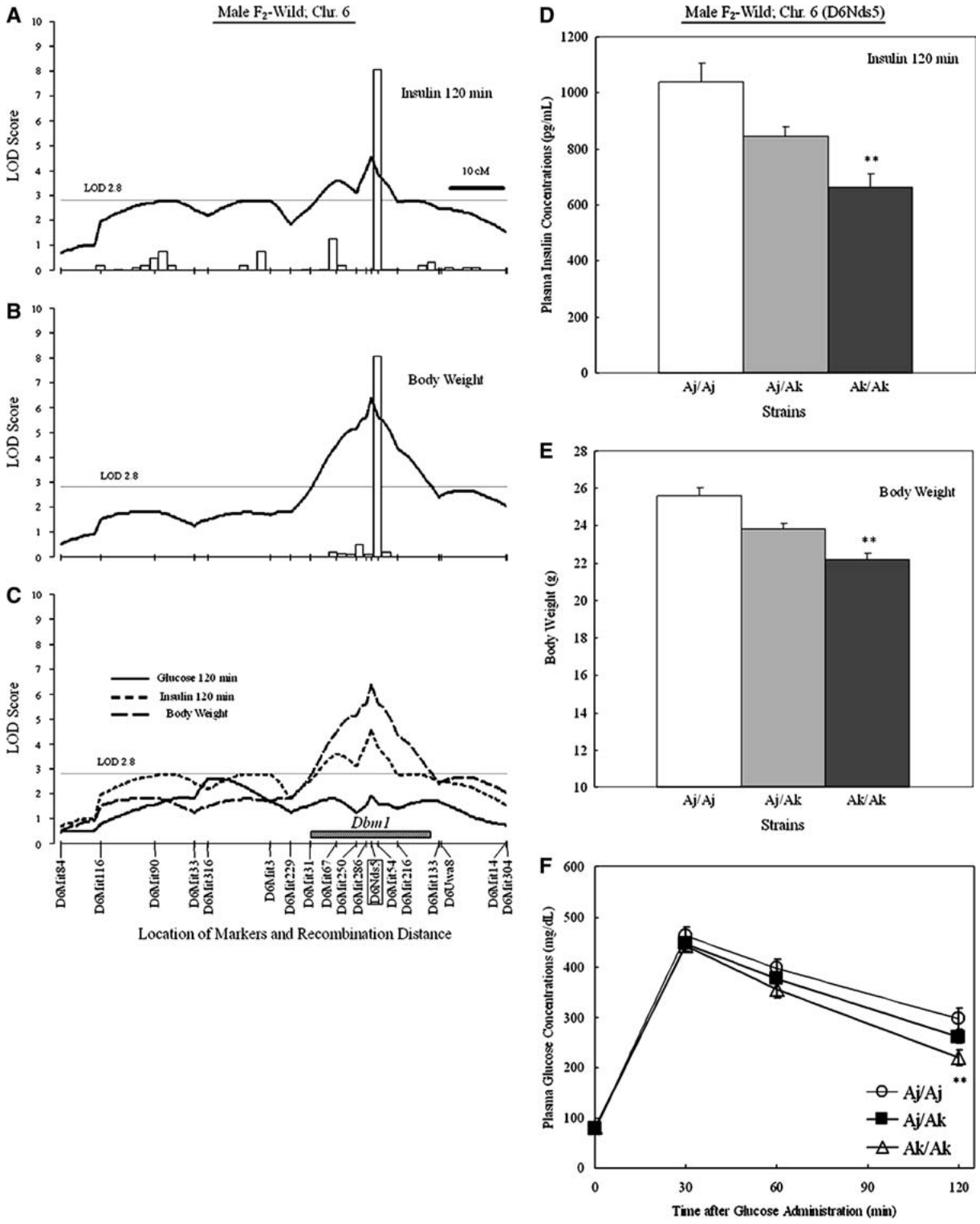
Fig. 1d. Plasma glucose concentrations in mice homozygous for the A/J allele (Aj/Aj) were higher than those in mice homozygous for the Akita allele (Ak/Ak) at all time points during IPGTT. As for insulin, no linkage was observed at this locus (LOD < 1) and there was no significant difference between both homozygous allele groups in plasma insulin concentrations at any time point ($p > 0.05$, data not shown). However, no linkages on Chromosome 6 affecting any trait were observed in female F_2-Hetero mice.

A significant linkage was also found at *D6Nds5* in male F_2-Wild mice. This linkage was shown with both plasma insulin concentrations at 120 min during IPGTT and BW, with maximum LOD scores of 4.52 and 6.32, respectively (Fig. 2). This region accounted for 11% and 16% of the phenotypic trait variance, respectively. Both LOD score plots showed sharp peaks and strong linkages with the traits, also supported by bootstrap analysis of the locus (Fig. 2a, b). Permutation testing showed that threshold LOD values for significant QTL for plasma insulin concentrations at 120 min and BW in male F_2-Wild mice were 3.84 and 3.87, respectively, based on 1000 permutations. The LOD score for plasma insulin concentrations at this locus was not significantly increased but rather was decreased from 4.52 to 1.82 when analyzed using BW as an interactive covariate (Supplementary Fig. 2c). We also identified a weak linkage near *D6Nds5* for plasma glucose concentrations at 120 min during IPGTT, with a LOD score of 1.93 (Fig. 2c). In this region, the A/J allele was associated with higher insulin concentrations (Fig. 2d), higher BW (Fig. 2e), and weaker glucose clearance after glucose administration (Fig. 2f) compared to the Akita allele. These tendencies were consistent with the QTL analysis of male F_2-Hetero mice (Fig. 1d). We designated this QTL detected in both F_2-Hetero and F_2-Wild mice as *Dbm1* (**di**a**b**etic **m**odifier QTL No. 1). No suggestive linkages were found on Chromosome 6 in female F_2-Wild mice.

Dbm2 on Chromosome 11. The second QTL affecting plasma glucose concentrations in male F_2-Wild mice was identified on Chromosome 11. The region close to the markers *D11Mit254* and *D11Mit203* showed a maximum LOD score of 2.45 for fasting plasma glucose concentrations (Fig. 3a), and overlapping significant linkages were observed for plasma glucose concentrations at 30, 60, and 120 min near *D11Mit254*, with peak LOD scores of 3.69, 4.50, and 5.78, respectively (Fig. 3b, c). This locus accounted for 6%, 9%, 11%, and 14% of the phenotypic variance, respectively. Bootstrap analysis supported the LOD score peak at *D11Mit254*. We called this locus *Dbm2*. Permutation testing showed threshold LOD score values for significant QTL in male F_2-Wild mice of 3.75, 3.67, 3.69, and 3.84 for plasma glucose concentrations at 0, 30, 60, and 120 min, respectively, based on 1000 permutations. The permutation thresholds for a suggestive linkage with these traits were 2.26, 2.24, 2.28, and 2.28, respectively. The LOD score of 5.78 was almost the same in the QTL analysis using BW as an interactive covariate with a LOD score of 6.30 for plasma glucose concentrations at 120 min during IPGTT (Supplementary Fig. 2b). The Akita-derived alleles in this region were associated with higher plasma glucose concentrations and decreased glucose clearance after glucose administration (Fig. 3d), and there was no significant difference between both homozygous allele groups in plasma insulin concentrations at any time point ($p > 0.05$, data not shown). In female F_2-Wild mice, no suggestive linkages with any of the traits examined were observed on Chromosome 11.

Fig. 2. LOD score plots for overlapping QTL on Chromosome 6 (*Dbm1*) and plasma glucose and insulin concentrations during IPGTT and BW according to *D6Nds5* genotype in male F_2-Wild mice. (**a**–**c**) The horizontal line indicates the threshold LOD score value of 2.8 for a suggestive linkage. The short bold line shows a genetic distance of 10 cM. The location of the marker showing the maximum LOD score in this analysis, *D6Nds5*, is boxed. LOD score plots and bootstrap analysis are shown for plasma insulin concentrations at 120 min during (**a**) IPGTT and (**b**) BW. (**c**) LOD score plots for plasma glucose concentrations at 120 min (solid line), plasma insulin concentrations at 120 min (broken line), and BW (dashed line). (**d, e**) Plasma insulin concentrations at 120 min during (**d**) IPGTT and (**e**) BW are shown. White columns denote mice homozygous for the A/J allele (Aj/Aj, $n = 36$). Gray columns denote heterozygous mice (Aj/Ak, $n = 93$). Black columns denote mice homozygous for the Akita allele (Ak/Ak, $n = 42$). Plasma glucose concentrations after fasting for 16 h during IPGTT are shown (**f**). Open circles denote mice homozygous for the A/J allele. Filled squares denote heterozygous mice. Open triangles denote mice homozygous for the Akita allele. Data shown as mean ± SEM. Statistical significance was shown for $p < 0.05$ * and $p < 0.01$ ** between the Ak/Ak and Aj/Aj genotypes.

Dbm3 on Chromosome 14. We found the third QTL, called *Dbm3*, on Chromosome 14 in male F_2-Hetero mice. It showed a suggestive linkage between plasma glucose concentrations and a Chromosome 14 locus between the markers *D14Mit98* and *D14Mit82* (14.8 cM). The region around the *D14Mit207* and *D14Mit202* markers showed a maximum LOD score of 4.17 for fasting plasma



glucose concentrations (Fig. 4a, c), 3.41 for plasma glucose concentrations at 30 min (Fig. 4b, c), 2.26 for plasma glucose concentrations at 60 min, and 2.02 for plasma glucose concentrations at 120 min during IPGTT. The LOD score of 4.17 for fasting plasma glucose concentrations was not changed in the QTL analysis using BW as an interactive covariate with LOD score of 5.02 (Supplementary Fig. 2a). The

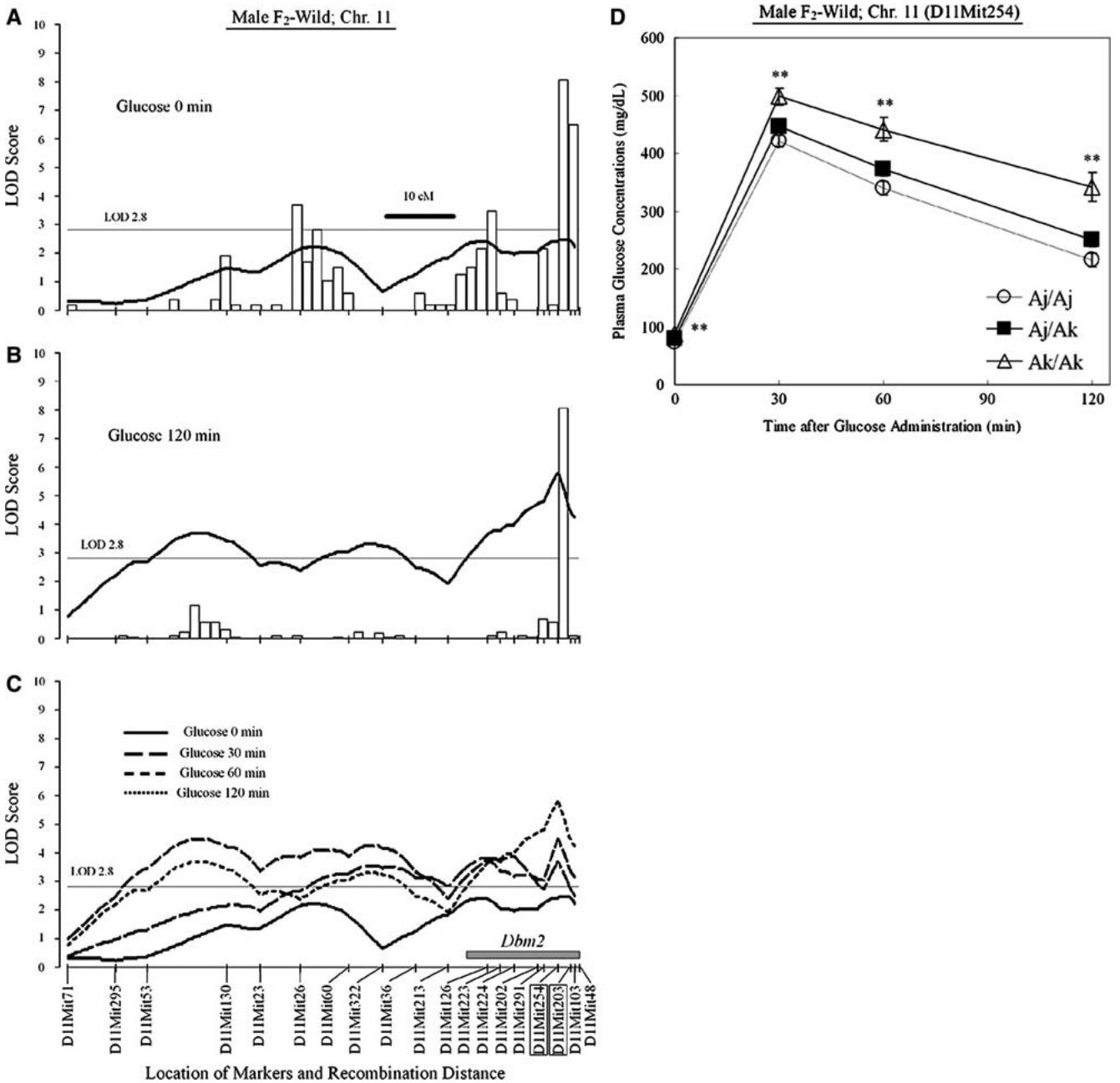


Fig. 3. LOD score plots for overlapping QTL on Chromosome 11 (*Dbm2*) and the plasma glucose concentrations during IPGTT according to *D11Mit254* genotype in male F_2-Wild mice. (**a**–**c**) The horizontal line indicates the threshold LOD score value of 2.8 for a suggestive linkage. The short bold line shows a genetic distance of 10 cM. The location of markers showing the maximum LOD scores in this analysis, i.e., *D11Mit254* and *D11Mit203*, are boxed. LOD score plots and bootstrap analysis are shown for plasma glucose concentrations at (**a**) 0 and (**b**) 120 min during IPGTT. (**c**) LOD score plots for plasma glucose concentrations at 0 (solid line), 30 (broken line), 60 (dashed line), and 120 min (dotted line). (**d**) Plasma glucose concentrations after 16 h of fasting during IPGTT. Open circles denote mice homozygous for the A/J allele (Aj/Aj, $n = 53$). Filled squares denote heterozygous mice (Aj/Ak, $n = 83$). Open triangles denote mice homozygous for the Akita allele (Ak/Ak, $n = 33$). Data shown as mean ± SEM. Statistical significance was shown for $p < 0.05$ * and $p < 0.01$ ** between the Ak/Ak and Aj/Aj genotypes.

Akita-derived alleles at *D14Mit207* showed high plasma glucose concentrations (Fig. 4d), and there was no significant difference between both homozygous allele groups in plasma insulin concentrations at any time point during IPGTT ($p > 0.05$, data not shown).

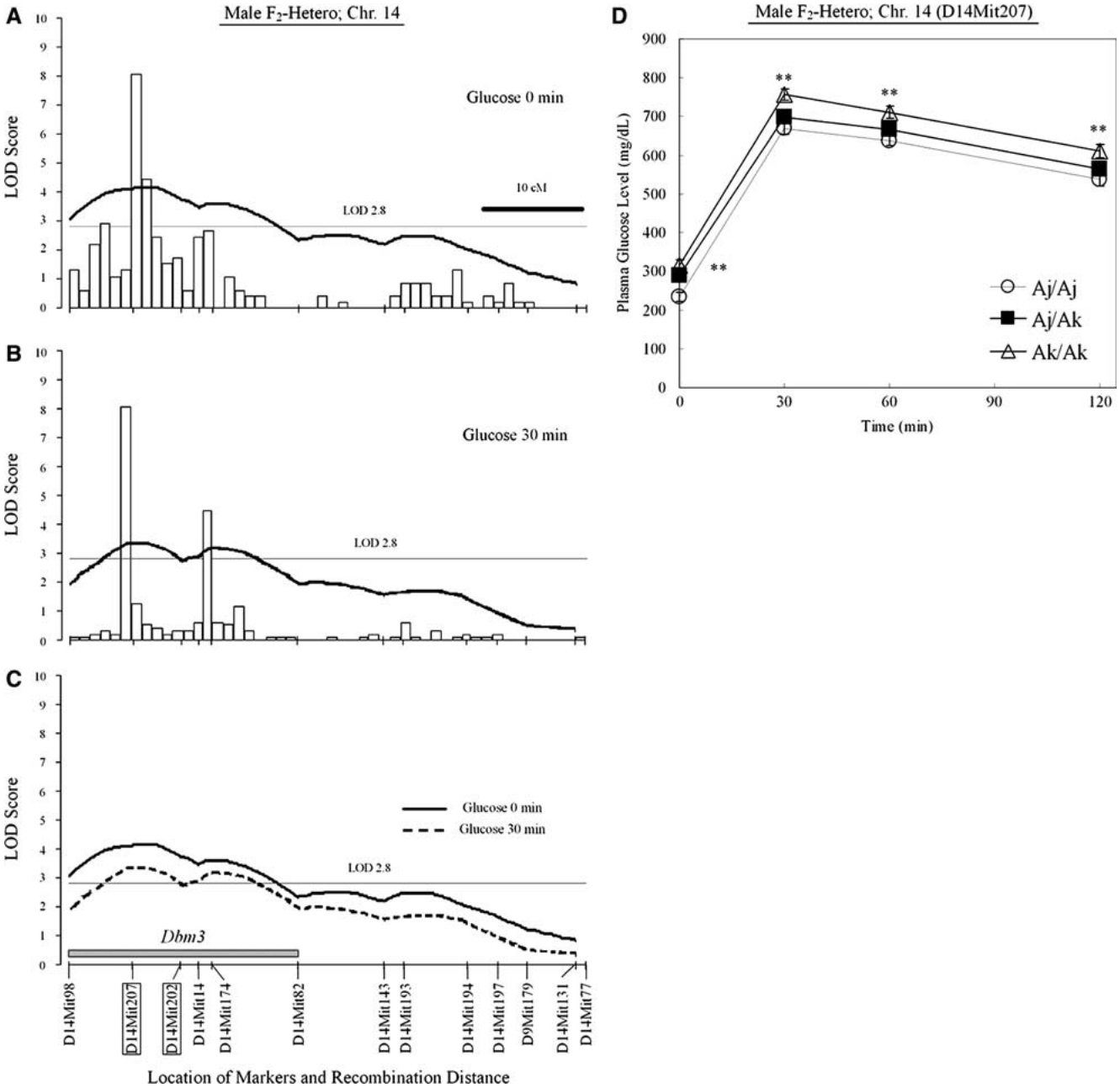


Fig. 4. LOD score plots for overlapping QTL on Chromosome 14 (*Dbm3*) and the plasma glucose concentrations during IPGTT according to *D14Mit207* genotype in male F_2-Hetero mice. (**a**–**c**) The horizontal line indicates the threshold LOD score value of 2.8 for a suggestive linkage. The short bold line shows a genetic distance of 10 cM. The location of markers showing the maximum LOD scores in this analysis, *D14Mit207* and *D14Mit202*, are boxed. LOD score plots and bootstrap analysis for plasma glucose concentrations at (**a**) 0 and (**b**) 30 min during IPGTT are shown. (**c**) LOD score plots for plasma glucose concentrations at 0 (solid line) and 30 min (broken line). (**d**) Plasma glucose concentrations after 16 h of fasting during IPGTT. Open circles denote mice homozygous for the A/J allele (Aj/Aj, n = 86). Filled squares denote heterozygous mice (Aj/Ak, n = 164). Open triangles denote mice homozygous for the Akita allele (Ak/Ak, n = 67). Data shown as mean ± SEM. Statistical significance was shown for $p < 0.05$ * and $p < 0.01$ ** between the Ak/Ak and Aj/Aj genotypes.

Dbm4 on Chromosome 15. The fourth QTL with significant linkage to plasma glucose concentrations was observed on Chromosome 15 in male F_2-Hetero mice. A locus with a maximum LOD score of 6.17, close to the *D15Mit233* and *D15Mit63* markers, affected fasting plasma glucose concentrations (Fig. 5a, c) and accounted for 8% of the phenotypic variance. Bootstrap analysis confirmed this

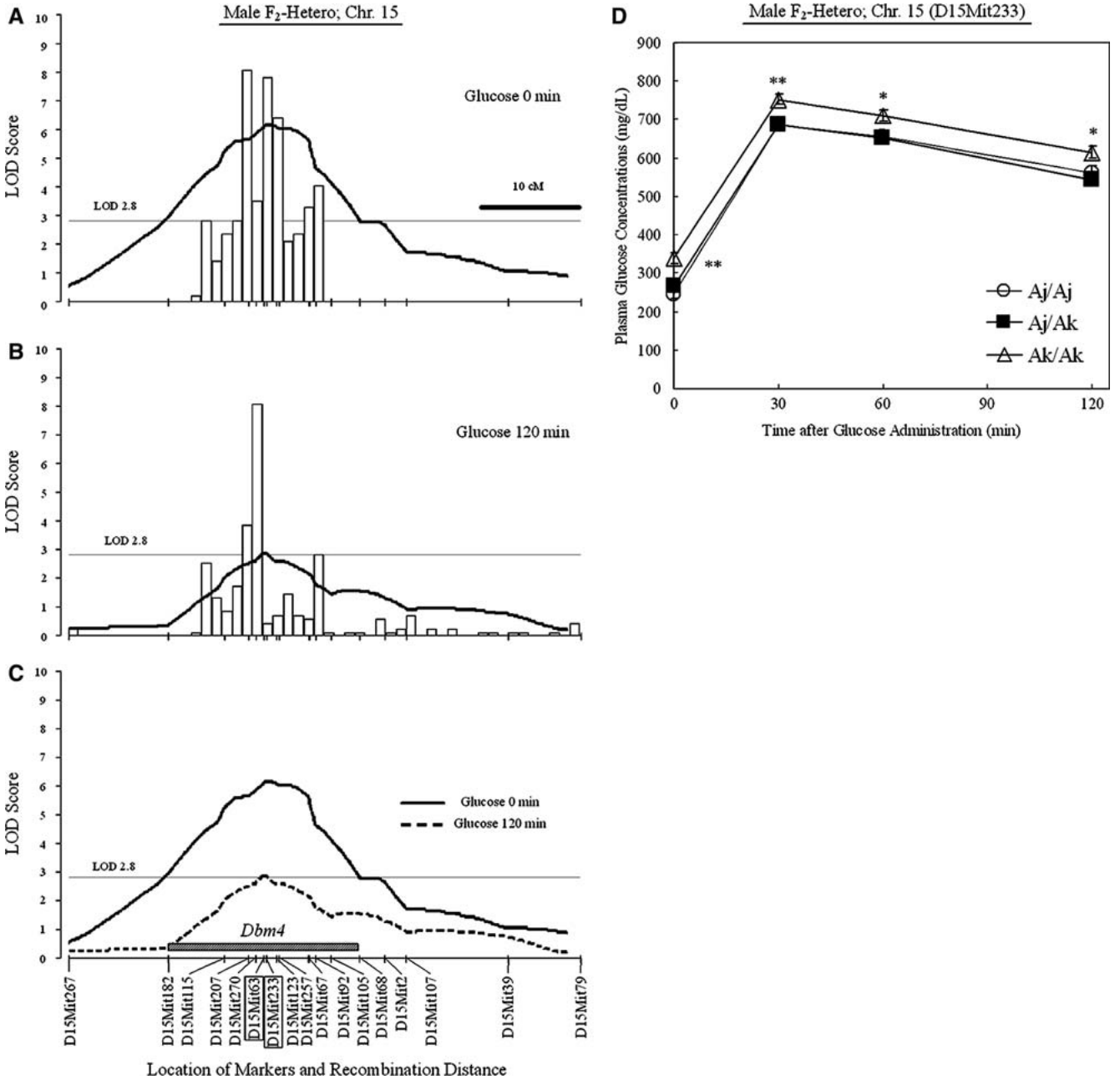


Fig. 5. LOD score plots for overlapping QTL on Chromosome 15 (*Dbm4*) and the plasma glucose concentrations during IPGTT according to *D15Mit233* genotype in male F_2-Hetero mice. (**a**–**c**) The horizontal line indicates the threshold LOD score value of 2.8 for a suggestive linkage. The short bold line shows a genetic distance of 10 cM. The location of markers showing the maximum LOD scores in this analysis, *D15Mit233* and *D15Mit63*, are boxed. LOD score plots and bootstrap analysis for plasma glucose concentrations at (**a**) 0 and (**b**) 120 min during IPGTT are shown. (**c**) LOD score plots for plasma glucose concentrations at 0 (solid line) and 120 min (broken line). (**d**) Plasma glucose concentrations after 16 h of fasting during IPGTT. Open circles denote mice homozygous for the A/J allele (Aj/Aj, n = 79). Filled squares denote heterozygous mice (Aj/Ak, n = 156). Open triangles denote mice homozygous for the Akita allele (Ak/Ak, n = 84). Data shown as mean ± S.E.M. Statistical significance was shown for $p < 0.05$ * and $p < 0.01$ ** between the Ak/Ak and Aj/Aj genotypes.

peak in a LOD score plot with a peak to within 5 cM relative to the LOD score peak. We called this locus *Dbm4*. A suggestive QTL was observed at the same locus, with a LOD score of 2.91 for the trait of plasma glucose concentrations at 120 min during IPGTT (Fig. 5b, c), which was confirmed by bootstrap analysis. Both traits of fasting and 120 min plasma glucose concentrations could be considered

phenotypic markers of insulin resistance in the presence of unchanged plasma insulin concentrations (see below). The LOD scores at *Dbm4* for plasma glucose concentrations by IPGTT at 30 and 60 min were 3.24 and 2.37, respectively. The LOD score of 6.17 was not changed in the QTL analysis using BW as an interactive covariate with LOD score of 8.06 (data for fasting plasma glucose concentrations is shown in Supplementary Fig. 2a). The Akita allele at *D15Mit233* was associated with higher plasma glucose concentrations at all time points during IPGTT (Fig. 5d), and there was no significant difference between both homozygous allele groups in plasma insulin concentrations at any time point during IPGTT ($p > 0.05$, data not shown). In female F_2-Hetero mice, fasting plasma glucose concentrations showed a weak linkage to *Dbm4* with a LOD score of 2.08, with the higher fasting plasma glucose concentrations observed for the Akita alleles than those for the A/J alleles (data not shown).

Other QTLs. In addition to the four major QTLs described above that significantly affected multiple traits, several other QTLs affecting single traits were also identified on Chromosomes 1, 3, 10, and 13. On Chromosome 1, a region near *D1Mit458* affected plasma insulin concentrations at 120 min during IPGTT with a LOD score of 4.92 in male F_2-Wild mice (Supplementary Fig. 2c), with the A/J alleles showing increased plasma insulin concentrations (data not shown). Regions near *D3Mit7* on Chromosome 3 and *D13Mit315* on Chromosome 13 showed a significant linkage with BW, with LOD scores of 4.60 and 4.73, respectively, in male F_2-Hetero mice and 7.58 and 6.56, respectively, in female F_2-Hetero mice. While Akita alleles showed heavier BW than A/J allele in each case, there were no other effects on the other diabetes-related traits at *D3Mit7* and *D13Mit315* (data not shown). Chromosome 10 contained a region near *D10Mit35* that affected only fasting plasma glucose concentrations with a LOD score of 5.28 in male F_2-Hetero mice (Supplementary Fig. 2a), with the Akita alleles showing increased plasma glucose concentrations (data not shown). We examined pairwise interactions among the QTLs we identified, but no suggestive interactions were detected (data not shown).

Discussion

The Akita mouse strain was established from the B6 mouse strain as a model of spontaneous diabetes with hypoinsulinemic hyperglycemia (Yoshioka et al. 1997; Kayo and Koizumi 1998; Wang et al. 1999). Akita mice exhibit sex-dependent diabetes-related phenotypes, with severe and mild diabetes in male and female mice, respectively (Yoshioka et al. 1997). The A/J mouse strain, used for the cross experiment in our study, is genetically different from the B6 strain, because a high-fat diet induced insulin resistance in B6 mice but not in A/J mice (Surwit et al. 1991; Kayo et al. 2000). As shown in Table 1, phenotypes including plasma glucose and insulin concentrations and BW in F_2-Hetero and F_2-Wild mice were different from those of the Akita, B6, and A/J strains.

Standard deviations of the measured phenotypes, including plasma glucose and insulin concentrations and BW in both F_2-Hetero and F_2-Wild mice were greater than those in the parental strains (Table 1, Supplementary Fig. 1). F_2-Hetero mice carrying the *Ins2* gene mutation showed lower fasting plasma glucose concentrations and higher plasma insulin concentrations at 120 min during IPGTT compared to Akita mice. These observations strongly suggested that the diabetes-related phenotypes in F_2-Hetero mice were consistent with high heritability of these traits. In addition, plasma glucose and insulin concentrations at 120 min in male F_2-Wild mice were significantly higher than those in A/J or B6 mice, and BW of male and female F_2-Wild mice was greater than that of A/J or B6 mice. As the phenotype analysis of F_2 progeny can be applied to QTL analysis, we attempted to find novel genetic modifiers that changed T2D-related traits. In our study, we identified several modifier QTLs that affected T2D-related traits in both hypoinsulinemic diabetic F_2-Hetero mice and normoinsulinemic nondiabetic F_2-Wild mice.

The first QTL, *Dbm1*, was identified on Chromosome 6 and was suggestively associated with plasma glucose concentrations at 0 and 120 min after glucose administration in male F_2-Hetero mice (Fig. 1), and also significantly associated with insulin concentrations at 120 min and BW in male F_2-Wild mice (Fig. 2). The presence of two peaks in the LOD score plot for male F_2-Hetero mice (Fig. 1a, b) suggested the possibility of two modifiers in this region. The suggestive LOD score at *D6Nds5* for plasma glucose concentrations at 0 and 120 min during IPGTT in male F_2-Hetero mice (Fig. 1) coincided with significant LOD score peaks at the same marker locus for plasma insulin concentrations at 120 min and BW in male F_2-Wild mice (Fig. 2). As for the coincidental LOD peaks for BW and plasma insulin concentrations in F_2-Wild mice (Fig. 2c), we performed QTL analysis on plasma insulin concentrations using BW as an interactive covariate, but no significant change in LOD scores was detected (Supplementary Fig. 2c). This suggested that the

significant LOD profile for plasma insulin concentrations did not depend on BW and that the single locus *Dbm1* could affect both plasma insulin concentrations and BW. That is, plasma insulin concentrations did not interact but instead tended to correlate with BW in all of male F_2-Wild mice (R = 0.45) as shown in the scatterplot (Supplementary Fig. 3). Plasma insulin concentrations correlated with BW at *Dbm1*, where BW could represent lean or fat body mass. This BW-associated locus is assumed to be associated with insulin resistance for the following reasons. The A/J allele at *Dbm1* (*D6Nds5*) showed increased plasma glucose concentrations (Figs. 1d, 2f) without changing plasma insulin concentrations in male F_2-Hetero mice but showed increased plasma insulin concentrations in male F_2-Wild mice (Fig. 2d). The increased plasma glucose concentrations in the presence of unchanged or increased plasma insulin concentrations suggest the presence of further modifier(s) at the *Dbm1* locus that affect insulin resistance. Also, the overlapping of modifier QTLs at the same chromosomal location in F_2-Hetero and F_2-Wild mice for multiple traits suggested the presence of strong modifier genes functioning irrespective of the presence or absence of the heterozygous *Ins2* mutation.

A similar QTL has been reported on Chromosome 6 for the SMXA recombinant inbred strain (Kobayashi et al. 2003; Anunciado et al. 2001) and for the NSY strain, a model of spontaneous T2D established from outbred ICR mice (Ueda et al. 1999). Kobayashi et al. (2003) identified a QTL near *D6Mit287*, close to *D6Nds5* (about 2.5 Mb/2.0 cM), that showed increased plasma glucose concentrations and BW for the A/J allele compared to the SM/J allele. Ueda et al. (1999) also identified *Nidd3nsy* on Chromosome 6 that affected plasma glucose and insulin concentrations and visceral fat weight. This *Nidd3nsy* locus, located between the *D6Mit209* and *D6Mit52* markers, spanned a region of about 35 Mb, including *D6Nds5*, and may contain several other modifiers. These data support the idea that the pleiotropic effects on plasma glucose and insulin concentrations and on BW may be to the result of several modifier genes located in a small region. The region near the *Dbm1* locus on mouse Chromosome 6, which corresponds to human Chromosome 3p26, contains several candidate genes, including contactin 4 (*Cntn4*), interleukin 5 receptor α (*Il5ra*), and inositol 1,4,5-triphosphate receptor 1 (*Itpr1*). However, their possible roles in T2D remain unclear. A single nucleotide polymorphism in the peroxisome proliferator-activated receptor γ (*Pparg*) gene, which results in a Pro12Ala polymorphism, is related to human obesity and T2D (Koch et al. 1999; Stumvoll and Häring 2002). Because *Pparg* is also located on mouse Chromosome 6 near the *D6Mit54* and *D6Mit216* markers and because it is separated from *D6Nds5* by about only 6.0 Mb, *Pparg* should also be considered a candidate gene.

The second QTL, *Dbm2*, on Chromosome 11 affected plasma glucose concentrations as measured by IPGTT in male F_2-Wild mice (Fig. 3) without affecting insulin concentrations. Thus, in the region near *D11Mit254*, the Akita-derived alleles apparently induced insulin resistance in nondiabetic mice. A similar LOD score plot with respect to plasma glucose concentration was reported for the TSOD diabetic mouse (Hirayama et al. 1999). This TSOD mouse locus (*D11Mit128*), named *Nidd4*, is separated from *D11Mit254* by about only 1.0 Mb, which suggests the presence of a potential genetic modifier of T2D around *Dbm2*. The region near *Dbm2* on mouse Chromosome 11, which corresponds to human 17q24-25, includes genes such as somatostatin receptor type 2 (*Sstr2*), G protein-coupled receptor 142 (*Gpr142*), galactokinase 1 (*Galk1*), acyl-Coenzyme A oxidase 1 (*Acox1*), and as several unknown genes.

The third QTL, *Dbm3*, identified in our study also overlapped with regions recognized in previous reports. The *Dbm3* affected plasma glucose concentrations because it was located near *D14Mit207* on Chromosome 14, similar to the QTL reported for the NSY diabetic mouse model (Ueda et al. 1999) and mice heterozygous for deletion of both the insulin receptor and IRS-1 (Almind et al. 2003). Because this 14.8-cM (12.5-Mb) region, corresponding to human 3p14, 3p24, 6p21, and 10q22, is very broad, further dense linkage analysis using congenic mice is required to identify the modifier genes within this region.

The fourth QTL was identified on Chromosome 15 and was named *Dbm4*. *Dbm4* showed a significant linkage with fasting plasma glucose concentrations and a suggestive linkage with plasma glucose concentrations at 120 min during IPGTT in male F_2-Hetero mice (Fig. 4). This T2D-related trait suggested that insulin resistance and the Akita allele at *Dbm4* (*D15Mit233*) increased plasma glucose concentrations at all time points during IPGTT. A weak linkage that affected fasting plasma glucose concentrations was also found at *Dbm4* in female F_2-Hetero mice (LOD = 2.08), although no linkage was identified in male or female F_2-Wild mice. Because plasma insulin concentrations were not altered, the Akita-derived alleles at *Dbm4* appeared to confer insulin resistance that led to fasting hyperglycemia and hyperglycemia in response to glucose administration. Similar QTL analysis results were reported

for TSOD mice, a T2D model established from the outbred ddY strain (Hirayama et al. 1999). TSOD mice showed a suggestive linkage between plasma glucose concentrations and *D15Mit63*, relatively close to our LOD score peak at *D15Mit233*, with a recombination distance of about 0.3 cM. These findings support the existence of a diabetic modifier gene in this region. Genes around *Dbm4* on mouse Chromosome 15, which corresponds to human Chromosome 8q24, include adenylate cyclase 8 (*Adcy8*), otoconin 90 (*Oc90*), thyroglobulin (*Tgn*), and sialyltransferase 4A (*Siat4a*) according to the Ensembl Mouse Genome Browser, but their relationship to T2D is currently unknown. Peroxisome proliferator-activated receptor α (*Ppara*) is also located on mouse Chromosome 15, but it is apparently not a candidate gene because it is separated by a physical distance of about 20 Mb from the *Dbm4* locus.

To discover genuine modifier genes, it is essential to produce congenic mice that retain nothing but the QTL in question, and this is currently underway in our laboratory. Polymorphism searches in the parental strains and detection of differentially expressed genes can also be used to identify the modifier genes, and further molecular analysis using congenic, transgenic, or knockout strains will help clarify the function of candidate modifier genes. The identification of novel modifiers in the mouse will supply candidate genes for human association studies to disclose modifier genes of human T2D.

In conclusion, we have discovered four major QTLs—*Dbm1*, *Dbm2*, *Dbm3*, and *Dbm4*—as modifier QTLs specifically affecting T2D-related traits after genome-wide analysis of F_2 progeny of hypoinsulinemic diabetic Akita mice crossed with nondiabetic A/J mice. *Dbm1* overlapped the QTLs in the SMXA recombinant inbred strain or the NSY mouse strain, and *Dbm2* and *Dbm4* overlapped the QTLs in the TSOD mouse strain in previous reports. We also found that these four diabetic modifier QTLs are conditional on heterozygous *Ins2* gene mutation and sex to exert their modifier functions. Identification of the genes responsible for QTLs detected in our study would potentially provide new drug development targets for human T2D.

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