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Identification of diabetes susceptibility loci in *db* mice by combined quantitative trait loci analysis and haplotype mapping

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Abstract

To identify the disease-susceptibility genes of type 2 diabetes, we performed quantitative trait loci (QTL) analysis in F_2 populations generated from a BKS.Cg-*m*+/+*Lepr*^{db} and C3H/HeJ intercross, taking advantage of genetically determined obesity and diabetes traits associated with the *db* gene. A genome-wide scan in the F_2 populations divided by sex and *db* genotypes identified 14 QTLs in total and 3 major QTLs on chromosome (Chr) 3 (LOD 5.78) for fat pad weight, Chr 15 (LOD 6.64) for body weight, and Chr 16 (LOD 8.15) for blood glucose concentrations. A linear-model-based genome scan using interactive covariates allowed us to consider sex- or sex-by *db*-specific effects of each locus. For the most significant QTL on Chr 16, the high-resolution haplotype comparison between BKS and C3H strains reduced the critical QTL interval from 20 to 4.6 Mb by excluding shared haplotype regions and identified 11 nonsynonymous single-nucleotide polymorphisms in six candidate genes.

Keywords: Type 2 diabetes (T2D); *Lepr*^{db} (*db*) mice; F_2 intercross; R/qtl; Conditional QTL; SNP (single-nucleotide polymorphism); Haplotype block; Haplotype mapping

Polygenic diseases such as type 2 diabetes (T2D) result from the interaction between multiple genetic and environmental factors. To dissect genetic determinants of polygenic diseases, quantitative trait loci (QTL) mapping in rodents has revealed many chromosomal regions affecting polygenic traits. In light of the polygenic basis of diabetes and/or obesity, it is believed that the effects of defined QTLs are influenced by genetic background [1]. Following Coleman's [2] demonstration that genetic modifiers influence diabetes in rodents, several QTLs [3–7] and modifier loci affecting obesity induced by monogenic genes [8–11] have been subsequently reported. To clarify the effects of sex or genotype for T2D-related phenotypes, it is necessary to analyze the results of a genome scan using both covariate-independent and -dependent approaches with genotype and/or sex as a covariate in F_2 subpopulations and in the combined F_2 population.

To dissect the QTLs that modify obesity-associated T2D phenotypes, we took advantage of genetically determined obesity and diabetes using the *db* mouse. We generated an F_2 intercrossing population between BKS.Cg- *m*+/+*Lepr*^{db} (BKS-*db*/−) and C3H/HeJ (C3H). The *db* mouse represents an animal model of human obesity associated with T2D [12,13]. Diabetes in a *db* mouse with a BKS background is severe; in contrast, it is mild in a mouse with a C57BL/6J (B6) background [14]. T2D-related phenotypes including body weight (BW) and blood glucose (BG) concentrations are remarkably diversified when *db* mice are crossed with other inbred strains of C3H, DBA/2J (DBA), B6, or 129/J [15,16]. The phenotypic diversification in these crossed progeny suggests the presence of susceptibility genes that affect T2D-related phenotypes under high BG concentrations or obesity.

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We divided the F_2 population into six subpopulations by sex and the genotype of *db*, i.e., those homozygous for the *db* (*db*/*db*) mutation (F_2-*db*/*db*), those heterozygous for the *db* (*db*/−) mutation (F_2-*db*/−), and those without *db* mutation (F_2- -/-; wild type). However, to clarify the sex- or *db*-specific effects of the QTLs, it is not sufficient to analyze two sexes or *db* genotypes separately and interpret the differences. This is due to the fact that a smaller sample size reduces the power to detect effects. Consequently, differences among subpopulations may be due to chance fluctuations in a setting where the power to detect QTLs is moderate or low. We overcame that problem by incorporating sex and *db* genotypes into genome scans as additive and interactive covariates. First, we analyzed a genome-wide scan in each subpopulation. Second, we analyzed the sex-specific effects of the QTLs in the F_2-*db*/*db* and F_2-*db*/− subpopulations with sex as a covariate. Finally, we examined sex-, *db*-, and sex-by-*db*-specific effects of the QTLs in the whole combined F_2 population (Fig. 1).

For the most significant QTL region on Chr 16, we compiled a comprehensive haplotype mapping and a set of potentially functional sequence variants between C3H and BKS. Additionally, we used phenotype-responsible gene databases that were identified by QTL analysis [17,18] to map the candidate genes. Here we show that the publicly available haplotype information effectively narrows the critical QTL interval and helps to detect candidate genes.

Results

Phenotypes in parental strains C3H and BKS and the F_2 populations

Mice homozygous for the *db* mutation (BKS-*db/db* and F_2-*db/db*) were characterized by higher BWs, BG concentrations, and fat pad weights compared to parental strains of BKS, C3H, and BKS or F_2 mice heterozygous for the *db* mutation (BKS-*db/−* and F_2-*db/−*) (Table 1). Although both the BKS-*db/db* and the F_2-*db/db* are obese, the BKS-*db/−*, F_2-*db/−*, and BKS remained nonobese and nondiabetic, suggesting that *db* is fully recessive.

BWs and BG concentrations were markedly different in comparisons between BKS-*db/db* and F_2-*db/db* or between BKS-*db/−* and F_2-*db/−*. BW in F_2-*db/db* was significantly increased compared to that in both male and female BKS-*db/db* at all examined ages ($p<0.0001$). BW in F_2-*db/−* was also significantly increased compared to that in BKS-*db/−* in both sexes ($p<0.0001$ for both) (Fig. 2A). Fat pad weight was

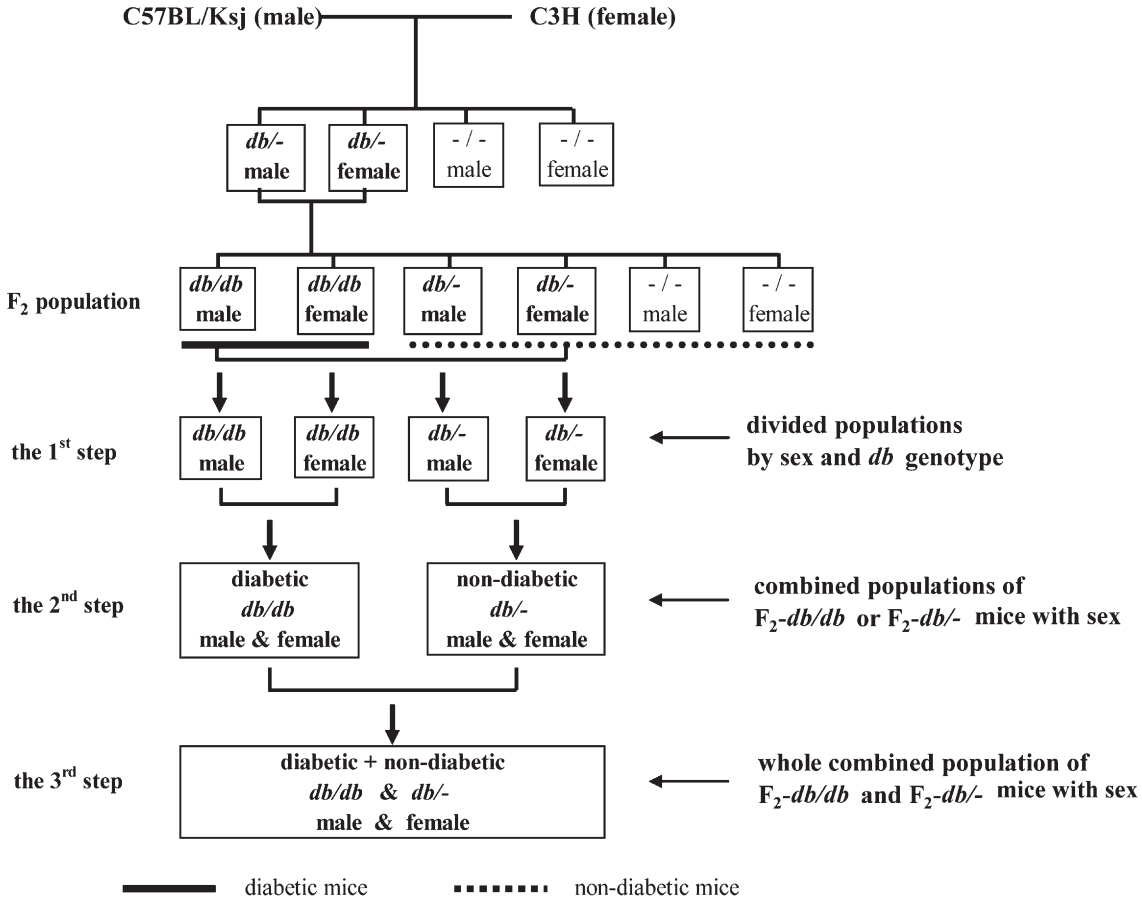

Fig. 1. Steps for detecting QTLs and verifying covariate-dependent effects. We divided the F_2 population into four subpopulations defined by sex and *db* genotype for the 1st step analysis. The 2nd step analysis was performed for the sex-dependent effects of the QTLs in F_2-*db/db* and F_2-*db/−* with sex as a covariate. Finally, we analyzed the genome scan only for the eight common phenotypes which were measured at the same time points in F_2-*db/db* and F_2-*db/−* in the whole combined F_2 population with sex and/or *db* genotype as a covariate.

Table 1
Comparison of phenotypes of the parental strain and F_2 populations

	Male					
	Parental				F_2	
Strain	C3H	BKS	BKS-*db/−*	BKS-*db/db*	F_2-*db/−*	F_2-*db/db*
Number of mice	18	19	15	19	168	118
BW (g)						
5W_nf	21.6±1.2	20.1±1.1	17.8±2.4	18.6±3.9	23.6±2.1	30.0±4.3
6W_nf	23.3±1.4	21.2±1.2	20.9±1.2	27.0±3.9	25.4±2.2	36.5±4.5
8W_nf	26.5±1.6	23.6±1.5	23.2±1.3	36.6±3.0	28.3±2.5	44.0±5.4
10W_nf	28.6±1.9	25.0±1.6	24.1±2.8		30.3±2.9	
Fat pad weight (mg)	430.7±145.3	287.1±120.4	387±35.3	1491.2±221.3	494.2±225.1	1927.3±375.2
BG (mM)						
4W_f	4.0±0.6		3.7±0.6	3.8±1.0	2.9±0.7	4.0±1.1
6W_f	4.2±0.6	3.8±0.6	3.5±0.3	6.4±1.2	3.6±0.7	6.6±2.2
8W_f	4.6±1.0	3.5±0.1	3.6±0.3	13.0±5.6	3.7±0.9	18.1±8.1
10W_f	6.0±1.2	4.2±0.6	3.8±0.8		5.8±1.4	
BG (mM)						
0 min in *ip* GTT	5.2±1.0	3.9±0.6	4.2±0.7	9.4±4.1	4.8±1.1	14.1±7.1
30 min in *ip* GTT	20.8±3.5	20.2±2.3	17.5±3.6	32.0±6.5	20.9±6.1	36.3±7.7
60 min in *ip* GTT	16.2±3.1	12.7±2.4	14.7±2.7	31.3±5.9	17.1±4.8	35.0±7.6
120 min in *ip* GTT	8.4±1.6	6.5±1.4	10.9±3.4	31.7±7.7	9.3±3.4	31.3±9.0
Insulin at 30 min in						
ip GTT (ng/dl)	1.1±0.3		0.8±0.5	7.0±5.0	1.1±0.5	3.3±1.8
Leptin (ng/ml)	1.2±1.0		0.2±0.5	14.2±5.4	1.4±2.3	19.7±9.3
TG (mg/dl)	95.1±56.9		79.4±24.7	109.9±25.0	98.9±47.5	154.3±74.9
	Female					
	Parental				F_2	
Strain	C3H	BKS	BKS-*db/−*	BKS-*db/db*	F_2-*db/−*	F_2-*db/db*
Number of mice	18	20	15	20	210	109
BW (g)						
5W_nf	18.1±1.5	16.9±0.8	16.1±1.4	16.8±2.8	20.0±1.8	28.0±4.3
6W_nf	19.2±1.8	17.3±0.9	17.8±1.0	24.4±2.9	20.9±1.9	34.8±4.5
8W_nf	21.1±1.8	18.2±1.0	18.8±1.1	34.1±2.3	22.6±2.2	43.8±5.1
10W_nf	22.7±2.0	19.8±1.0	20.4±1.1		24.3±2.6	
Fat pad weight (mg)	424.8±184.5	87.1±29.7	363.1±125.2	1919.9±304.7	489.2±249.0	2981.6±588.8
BG (mM)						
4W_f	4.0±0.9		2.7±0.5	3.5±0.9	2.6±0.6	3.8±1.0
6W_f	4.0±0.6	3.1±0.4	3.4±1.0	5.7±0.9	3.3±0.6	5.5±1.4
8W_f	4.5±1.0	2.8±0.4	3.2±0.4	12.0±6.4	3.4±0.8	13.6±5.7
10W_f	5.8±1.1	3.2±0.4	3.6±0.5		4.9±1.2	
BG (mM)						
0 min in *ip* GTT	4.7±0.7	3.8±0.9	3.8±0.6	10.5±3.2	4.1±0.8	10.8±4.3
30 min in *ip* GTT	13.1±2.6	17.0±3.4	12.7±2.4	28.9±6.4	14.3±4.4	34.6±8.0
60 min in *ip* GTT	11.4±1.9	10.2±2.1	12.1±2.8	31.5±7.1	12.5±3.1	33.8±8.3
120 min in *ip* GTT	6.2±1.1	5.3±0.1	7.4±1.6	33.0±9.2	6.5±1.7	31.7±9.0
Insulin at 30 min in						
ip GTT (ng/dl)	1.0±0.4		0.6±0.3	6.0±6.1	0.9±0.6	4.59±4.9
leptin (ng/ml)	1.9±1.5		1.0±1.4	14.8±3.7	2.0±2.4	22.3±10.9
TG (mg/dl)	91.9±60.3		69.8±20.6	100.3±20.2	95.1±52.1	114±41.4

nf, nonfasting; f, fasting.

significantly increased in F_2-*db/db* compared to BKS-*db/db* in both sexes ($p<0.0001$) (Fig. 2B).

BG concentrations at 0, 30, and 60 min in the *ip*GTT were significantly increased in male F_2-*db/db* ($p<0.05$), while serum insulin concentrations at 30 min in the *ip*GTT were significantly decreased ($p<0.0001$) compared to the BKS-*db/db*. In female F_2-*db/db,* BG concentrations were significantly increased only at 30 min in the *ip*GTT ($p<0.01$) compared to the BKS-*db/db*

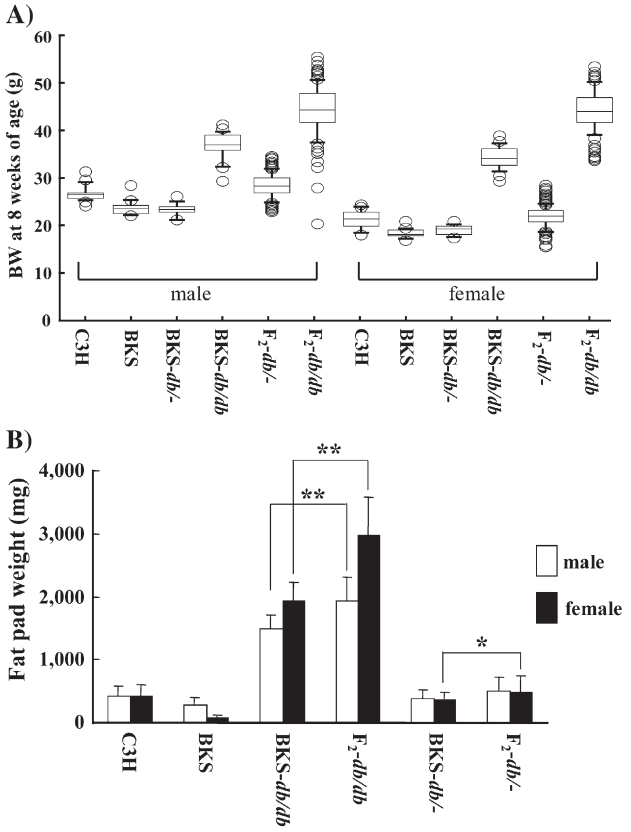

Fig. 2. Comparison of BW and fat pad weight in parental strains and F_2 populations. (A) Plot of BW of male and female parental strains (C3H, BKS, BKS-*db/−*, and BKS-*db/db*), F_2-*db/−*, and F_2-*db/db* at 8 weeks of age. (B) Fat pad weight is plotted for male (open column) and female mice (closed column) in parental strains and F_2 subpopulations. * or ** denotes significant difference at $p<0.05$ or $p<0.01$, respectively. Data presents box (median value±one quartile) and whisker plot above and below one quartile with the bars representing 10 and 90 percentiles.

(Table 1). BG concentrations at 30 and 120 min in the *ip*GTT were distributed more diversely in male F_2-*db/db* and F_2-*db/−* than those from male BKS-*db/db* or BKS-*db/−* (Supplementary Figs. 1A and 1B). These results indicate that the severity of the diabetes-related phenotypes was strongly dependent on the genetic background.

Genome-wide QTL scans in subpopulations divided by sex and db genotype

In this study, we analyzed genome-wide QTL scans in the subpopulations of F_2-*db/db* and F_2-*db/−* to elucidate the effect of *db* genotypes. We analyzed genome-wide QTL scans using R/qtl software [19,20] as an add-on package for the freely available statistical language R (http://www.r-project.org/) [21] by the EM algorithm. Significance thresholds ($\alpha=0.05$) were generated by permutation tests in each subpopulation (permutations=10,000) with R/qtl as described by Churchill [20,22].

In the first step, we performed the genome scan for BW at 9 weeks of age in the combined F_2 population including F_2-*db/db* and F_2-*db/−* mice. In this QTL analysis, the location corresponding to the *db* locus near the marker *D4Mit175* on Chr 4 was detected as a highly significant QTL with LOD=148 (Fig. 3A).

To map the QTLs responsible for the conditional effects, we next performed the genome scan in four subpopulations divided by sex and *db* genotype. The significant QTLs in four subpopulations are summarized in the left column in Table 2. In the male F_2-*db/db* subpopulation, four significant QTLs on Chr 2, 3, and 5 were identified. On Chr 2, two independent QTLs near *D2uc13pa* and *D2Mit307* showed linkage to BG concentrations and BW at 8 weeks of age and at sacrifice. Another QTL near *D3Mit86* showed strong linkage to fat pad weight but not to BW at any time point. However, these three loci did not exhibit any linkage in female F_2-*db/db*. On Chr 5, the QTL near *D5Mit356* showed linkage to BW at 7 weeks of age, but the peak of QTL of *D5Mit135* in females was separated by 2.6 cM from that in males.

In the male F_2-*db/−* subpopulation, four significant QTLs on Chr 9, 15, and 16 were identified. On Chr 9, two independent QTLs were observed: one QTL at *D8Mit112* exhibited linkage to BG concentrations only at 0 min in the *ip*GTT in male F_2-*db/−* and the other QTL affected fasting BG concentrations. The QTL at *D15Mit107* on Chr 15 showed continuous strong linkage to BW at 7, 8, 9, and 10 weeks of age and at sacrifice. The QTL in male F_2-*db/−* at *D16Mit81* on Chr 16 showed the strongest linkage to BG concentrations in the *ip*GTT at 30, 60, and 120 min. In contrast, no QTLs were found on Chr 9, 15, and 16 in female F_2-*db/−*. Of a total of 14 significant QTLs in F_2-*db/db* and F_2-*db/−* subpopulations, we selected 3 sex-dependent QTLs showing genetic effects with significant thresholds of $\alpha<0.001$ as the major QTLs: QTL on Chr 3 for fat pad weight (Fig. 3B), that on Chr 15 for BW (Fig. 3C), and that on Chr 16 for BG concentrations in the *ip*GTT (Fig. 4A).

Covariate-dependent QTL genome scans in combined population

Starting with the sex-dependent effect of the QTL that was first detected in the genome scan in every subpopulation, we next analyzed covariate-dependent scans with sex as an additive or interactive covariate. Most of the significant QTLs, especially the effects of QTL at *D16Mit81*, showed higher LOD scores with sex as an interactive covariate than those without interactive covariates, suggesting sex-dependent effects (Table 2). However, the only QTL near *D5Mit356* (*D5Mit135* in females) for BW in the *db/db* population showed little difference in LOD scores in two scan models, suggesting a sex-independent effect. The QTL at *D4Mit139* in the female *db/db* population also showed little difference in the two scan models, but this QTL did not reach the significance threshold in the genome scan in the male *db/db* population.

We further analyzed the covariate-dependent genome scans for the combined F_2 populations (F_2-*db/db* and F_2-*db/−*) with sex and *db* genotype. Data for assay items at the same time point in F_2-*db/db* and F_2-*db/−* (eight phenotypes) were analyzed only in the whole F_2 population (see Materials and methods). All of the QTLs analyzed in the whole combined F_2 population

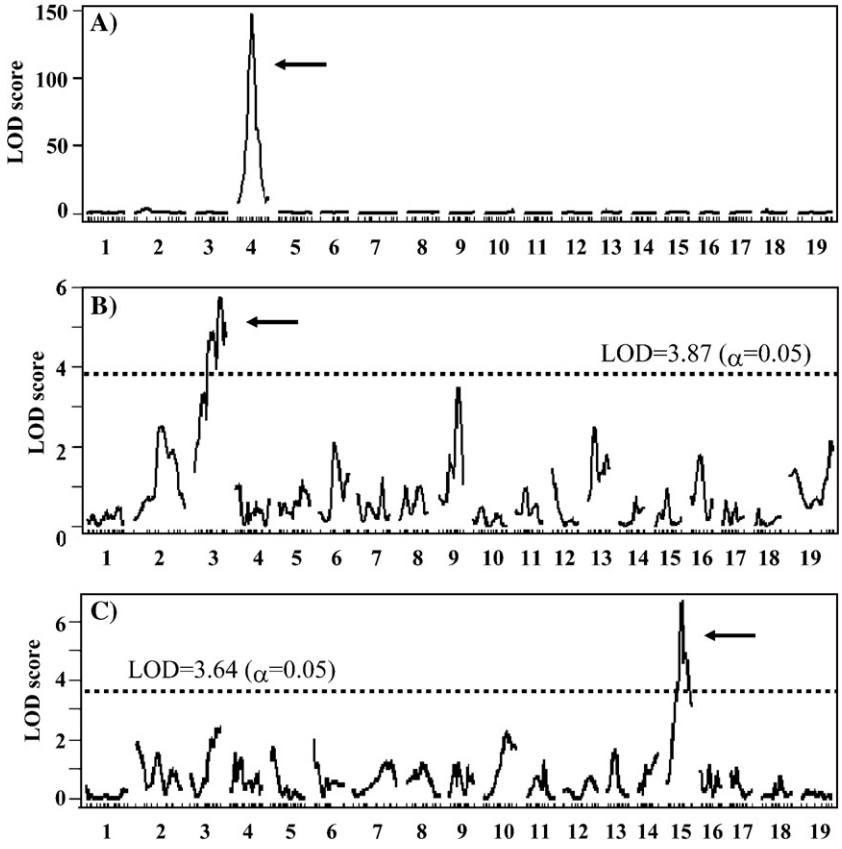

Fig. 3. Genome-wide QTL mapping. (A) Result of the genome scan for BW at 9 weeks of age in the combined F_2 population. The arrow indicates the *db* locus near the marker *D4Mit175* on Chr 4. (B) Result of the genome scan for fat pad weight in the male F_2-*db/db* subpopulation. (C) Result of the genome scan for nonfasting BW at sacrifice in male F_2-*db/−* subpopulation. The arrows indicate the locations of the significant QTLs on Chr 4 (A), Chr 3 (B), and Chr 15 (C).

showed higher LOD scores in the genome scans with interactive covariates than in those without interactive covariates (Table 2, right columns). These results suggest not only sex dependence but also *db*- and sex-by-*db*-dependent effects. However, the effect of QTL near *D5Mit356* (*D5Mit135* in female) was sex independent and *db* dependent.

Effect of genotype on phenotypes in the three significant QTLs

To evaluate genotype effects of the three significant QTLs ($\alpha = 0.001$), mice in each subpopulation were divided according to the genotype of the marker of the QTLs for BB (BKS homozygous), BC (BKS/C3H heterozygous), and CC (C3H homozygous) (Table 3). At *D3Mit86* on Chr 3, the BKS allele in this locus was associated with increased fat pad weight in a dominant manner. At *D15Mit107* on Chr 15, the C3H allele at this locus was associated with increased time-series BW in a dominant manner. At *D16Mit81* on Chr 16, the locus showed linkage to BG concentrations at 30, 60, and 120 min during the *ip*GTT, while the C3H allele at this locus was associated with increased BG concentrations in a dominant manner.

QTL on Chr 3, specific for fat pad weight but not for BW, was observed only in male F_2-*db/db*, and no QTLs were observed in male or female F_2-*db/−*. QTLs on Chr 15 for BW and on Chr 16 for multiple BG concentrations were observed only in male F_2-*db/−*. In both F_2-*db/db* and F_2-*db/−* subpopulations, QTLs were observed dominantly in one subpopulation, suggesting that they are highly conditional on sex and *db* genotype (Supplementary Fig. 2).

SNPs density, polymorphism for BKS vs C3H strains, and candidate genes by the interstrain cSNPs on Chr 16

We assumed that the interval between the telomere and *D16Mit81–D16Mit144–D16Mit101* (within 20 Mb in physical distance; Fig. 4B) represented the most significant QTL on Chr 16 because it showed the highest LOD scores for multiple phenotypes and the highest genotypic effects in a genome-wide scan. Polymorphisms (the differences in the genome sequence in each strain) can be used to map the QTL regions, because these variations correlate with phenotypic traits [23,24]. To define the QTL on Chr 16, we first searched the Celera RefSNP database for interstrain sequence variations between B6 and DBA strains. We adopted this approach because the genetic background of the C3H strain is similar to that of the DBA strain in this region on Chr 16 (see Genomics Institute of the Novartis Research Foundation [GNF] SNPview, (http://snp.gnf.org/) (Fig. 4D) [23]. We listed 212 SNPs between B6 and DBA by selecting 1 in every 50-kb interval (Fig. 4C, middle and bottom windows) of 7644 SNPs (Fig. 4C, closed columns in top

Table 2
Significant QTLs by a genome-wide scan

Genotype of *db*	Sex	Result of genome scan (covariate-independent)								Result of genome scan (covariate-dependent)					
		Locus			Divided F_2 populations by srx and *db* genotype					Combined population of F_2-*db/db* or F_2-*db/–* with sex		Whole combined population of F_2-*db/db* or F_2-*db/–* with sex			
		Chr	Marker[a]	cM	Phenotypes	Weeks of age at evaluations[b]	Maximum LOD	Level of significance (α)[a]	% Variance	LOD no covariate[c]	LOD sex[d]	LOD no covariate[e]	LOD sex[f]	LOD *db*[g]	LOD sex/*db*[h]
F_2-*db/db*	Male	2	*D2ucl3pa*	37.2	Non-fasting_BG	8	3.98	0.05	13	4.22	5.15	n.a.			
		2	*D2ucl3pa*	37.2	Fasting BG	9_s	4.53	0.01	15	4.22	6.22	n.a.			
		2	*D2Mit307*	91.3	Non-fasting_BW	8	4.23	0.05	13	2.81	4.61	2.95	3.9	7.6	8.3
		2	*D2Mit307*	91.3	Non-fasting_BW	9_s	4.22	0.05	13	3.09	4.46	3.05	3.67	7.9	8.4
		2	*D2Mit307*	91.3	Fasting_BW	9_s	4.02	0.05	13	2.91	4.3	n.a.			
		3	***D3Mit86***	70.2	Fad pad weight	9_s	5.78	**0.001**	17	2.8	4.1	n.a.			
		5	*D5Mit356*	32.3	Non-fasting_BW	7	3.61	0.05	11	4.68	5.1	3.56	4	9.85	10.2
	Female	4	*D4Mit139*	14.9	Leptin	9_s	5.3	0.01	17	4.3	4	n.a.			
		5	*D5Mit356*	32.3	Non-fasting_BG	9	3.72	0.05	11	3.6	4.12	n.a.			
		5	*D5Mit135*	34.9	Non-fasting_BW	9_s	3.63	0.05	13	5.32	5.61	3.61	3.73	10.7	10.8
		5	*D5Mit135*	34.9	Fasting_BW	9_s	3.88	0.05	14	5.67	5.98	n.a.			
F_2-*db/–*	Male	9	*D8Mit112*	10.9	*ip*GTT_pre	10	5.35	0.01	12	4.29	7.3	n.a.			
		9	*D9Mit208-D9Mit182*	35.8	Fasting_BG	6	4.9	0.01	10	3.27	5.9	n.a.			
		15	***D15Mit107***	35.8	Non-fasting_BW	7	4.49	0.01	10	2.9	5.23	3.35	4.5	4.36	5.1
		15	***D15Mit107***	35.8	Non-fasting_BW	8	6.21	**0.001**	14	4.16	7.3	3.5	4.8	3.8	5
		15	***D15Mit107***	35.8	Non-fasting_BW	9	5.61	**0.001**	13	3.79	6.26	2.59	4.13	3	4.2
		15	***D15Mit107***	35.8	Non-fasting_BW	10	6.38	**0.001**	15	4.36	7.28	n.a.			
		15	***D15Mit107***	35.8	Non-fasting_BW	11_s	6.64	**0.0001**	15	4.03	7.72	n.a.			
		15	*D15Mit107*	35.8	Fasting_BW	11_s	5.38	0.01	12	3.68	6.52	n.a.			
		16	***D16Mit81***	8	*ip*GTT_30 min	10	6.37	**0.0001**	15	4.28	8.7	n.a.			
		16	***D16Mit81***	8	*ip*GTT_60 min	10	8.15	**0.0001**	19	8.03	12.6	n.a.			
		16	***D16Mit81***	8	*ip*GTT_120 min	10	6.31	**0.001**	14	6.79	11.9	n.a.			
	Female	3	*D3Mit203-D3Mit21*	11.9	Non-fasting_BG	11	3.9	0.05	9	3.74	4.17	n.a.	n.a.		
		11	*D11Mit227*	2	*ip*GTT_60 min	10	3.7	0.05	8	5.7	6.3	n.a.			
		12	*D12Mit189*	33.8	Non-fasting_BW	6	3.72	0.05	8	2.04	3.7	1.07	1.2	6.43	6.6

[a] QTL that exceeded the significance thresholds ($\alpha < 0.01$) in covariate-independent are shown in boldface.
[b] Weeks of age at evaluation, s denotes at sacrifice point.
[c] Result of LOD value with sex as additive covariate (no interactive covariates).
[d] Result of LOD value with sex as additive and interactive covariates.
[e] Result of LOD value with sex and db genotype as additive covariates (no interactive covariates).
[f] Result of LOD value with sex and db genotype as additive covariates and sex as an interactive covariate.
[g] Result of LOD value with sex and db genotype as additive covariates and db genotype as an interactive covariate.
[h] Result of LOD value with sex and db genotype as additive and interactive covariates.

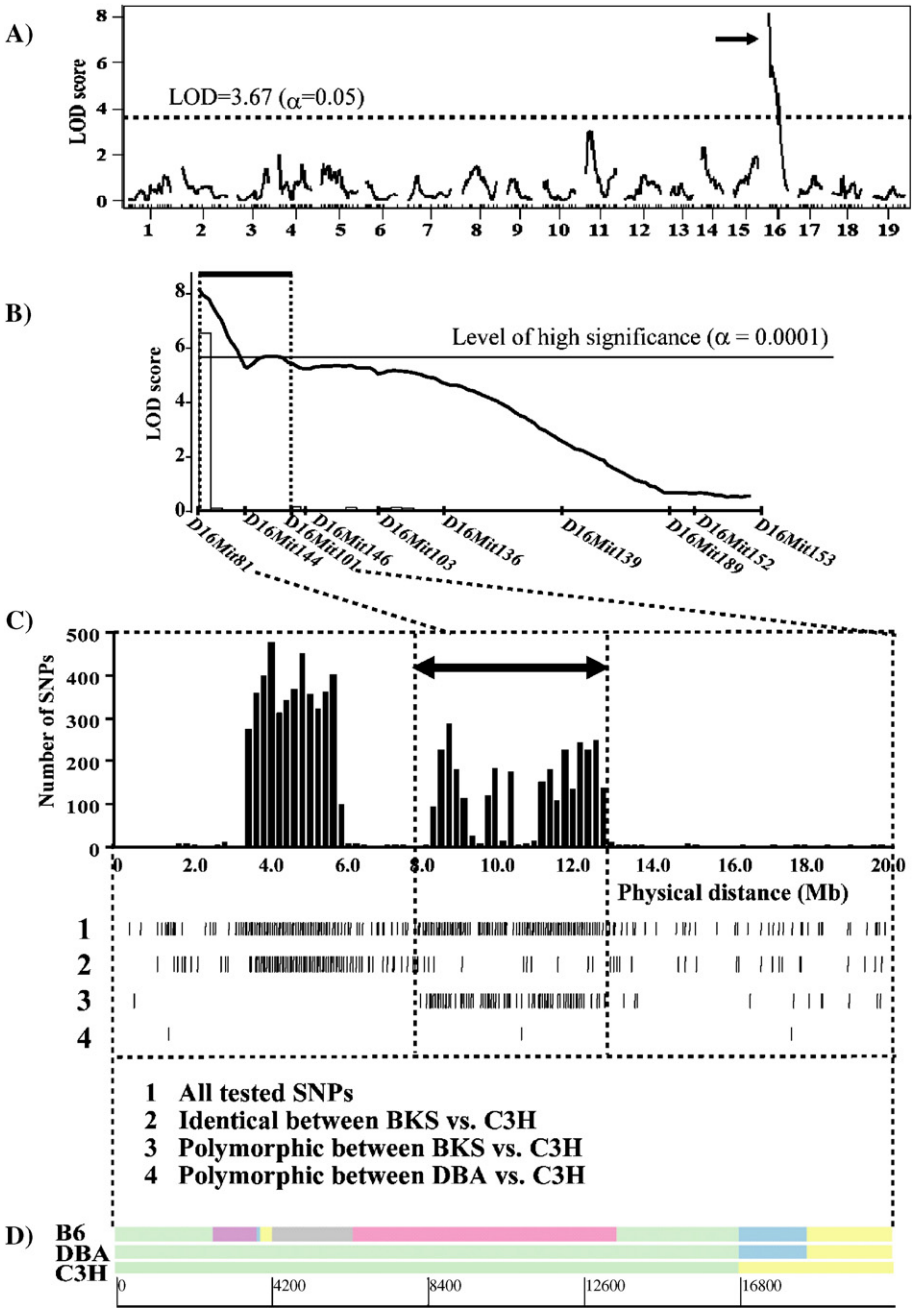

Fig. 4. High-resolution haplotype comparison between BKS and C3H strain. (A) Genome-wide QTL mapping result for BG concentrations at 60 min in the *ip*GTT in male F_2-*db*/− subpopulation. Arrow denotes the significant QTL on Chr 16. (B) Interval mapping result on Chr 16 for BG concentrations at 60 min in the *ip*GTT. (C) The top window represents number of SNPs in every bin of 200 kb against the physical distance spanning from 0 to 20 Mb from the telomere of Chr 16. In the middle and bottom windows, all 212 tested SNPs that are polymorphic between BKS and DBA are shown by vertical ticks (1), 108 identical SNPs (2), and 77 polymorphic SNPs (3) between BKS and C3H, and 2 polymorphic SNPs between C3H and DBA (4) by the reported sequence of the region spanning 20 Mb are shown. The bidirectional arrow denotes the critical QTL interval. (D) Haplotype structures of B6, DBA, and C3H strains in the target region on Chr 16 by GNF SNPview are shown. The shared haplotypes defined as regions in which three strains (B6, DBA, and C3H) share the same SNP alleles across multiple loci are indicated by the blocks with the same color, and different alleles are indicated by blocks with different colors in the horizontal bars.

window) in 20 Mb. We next examined by sequencing whether these 212 predicted polymorphisms are polymorphic between BKS and C3H. From all tested 212 target amplicons (Fig. 4C, 1), we obtained 187 high-quality sequences within the target region. From 187 predicted SNPs, 108 were identical (Fig. 4C, 2), 77 were polymorphic between BKS and C3H (Fig. 4C, 3), and 2 were polymorphic between C3H and DBA (Fig. 4C, 4). Based on these haplotype block results, we could exclude the locus for candidate genes between the telomere and 8 Mb and reduced the critical QTL interval to 4.6 Mb between the points at 8 and 12.6 Mb from 20 Mb by excluding shared haplotype regions.

Table 3
Comparison of phenotypes at 3 QTLs ($\alpha < 0.001$) in F_2-*db/db* or F_2-*db/−* according to the genotype of the marker of the QTLs

Genotype of *db*	Sex	Locus		Divided F_2 populations			Genotypes effect			*p* value		
		Chr	Marker	Phenotypes	Weeks of age at evaluations	LOD score	BB	BC	CC	BB vs. BC	CC vs. BC	BB vs. CC
							n=27	*n*=60	*n*=31			
F_2-*db/db*	Male	3	*D3mit86*	Fat pad weight (mg)	9_s	5.78	2120.5±269.7	1968.1±339.6	1686.4±401.6		<0.001	<0.0001
							n=44	*n*=84	*n*=40			
F_2-*db/−*	Male	15	*D15Mit107*	Non-fasting_BW (g)	7	4.49	26.1±2.2	27.2±2.1	28.4±2.4			<0.0001
			D15Mit107	Non-fasting_BW (g)	8	6.21	26.8±2.1	28.2±2.3	29.7±2.6	<0.01		<0.0001
			D15Mit107	Non-fasting_BW (g)	9	5.61	28.1±2.2	29.7±2.5	31.0±2.6	<0.01		<0.0001
			D15Mit107	Non-fasting_BW (g)	10	6.38	28.4±2.5	30.4±2.8	31.7±2.5	<0.01		<0.0001
			D15Mit107	Non-fasting_BW (g)	11_s	6.64	28.1±2.6	30.2±2.9	31.6±2.5	<0.01		<0.0001
			D15Mit107	Fasting_BW (g)	11_s	5.38	26.1±2.2	27.9±2.8	29.0±2.5	<0.01		<0.0001
							n=32	*n*=84	*n*=52			
		16	*D16Mit81*	BG in *ip*GTT_30 min	10	6.37	15.8±6.5	21.7±5.7	22.6±5.1	<0.001		<0.0001
			D16Mit81	BG in *ip*GTT_60 min	10	8.15	12.7±4.1	17.8±4.3	18.7±4.5	<0.001		<0.0001
			D16Mit81	BG in *ip*GTT_120 min	10	6.31	6.6±2.2	9.7±3.2	10.4±3.7	<0.001		<0.0001

Genotype abbreviations: BB: BKS homozygous, BC: BKS/C3H heterozygous, CC: C3H homozygous. Mice were grouped according to the marker that had a significant LOD score in F_2-*db/db* or a highly significant LOD score in F_2-*db/−*. Phenotypes are expressed as mean±SD.
p values denote the significance level in a classical Kruskal–Wallis test with post hoc tests using Scheffe's test for multiple comparisons.

We selected cSNPs accompanied by amino acid variation between B6 and DBA mice in this 4.6 Mb region based on the Celera database. These cSNPs were confirmed to be polymorphic between BKS and DBA. We searched the interstrain sequence differences and listed six polymorphic genes containing 11 mis-sense cSNPs between B6 and DBA (Table 4). Next, we sequenced to confirm polymorphic variations between BKS and C3H. All 11 SNPs were confirmed polymorphic between BKS and C3H. Knowledge-based ranking of candidate genes identified phenotype-responsible genes from the database. We searched the genes that could potentially explain phenotypes by the positional MEDLINE database (PosMed) from RIKEN (http://omicspace.riken.jp) [17,18]. This database suggests highly promising candidate genes in a given chromosomal interval based on document databases (e.g., MEDLINE) and published data to connect phenotypic functions. PosMed also finds significant genes within the hit documents with statistical tests and makes a ranked list of the genes. We identified two candidate genes, *Txndc11* and *MkL2* of six genes containing mis-sense cSNPs that were associated with phenotypes of BG concentrations, insulin, and other T2D-related phenotypes.

Discussion

Rodent models of human disease have been used to dissect the determinants of complex polygenic pathologies. Chief

Table 4
Candidate genes with mis-sense cSNPs between BKS and C3H in the critical QTL region on Chr 16

Symbol	Celera gene ID	Celera mis-sense SNP ID	Amino acid variation[b] (mis-sense mutation)	Results of polymorphisms				*p* value[a]
				B6	BKS	C3H	DBA	
D16Mit81								
Txndc11 (AI427833)	mCG126575	mCV22789937	Gln(CAG)826Arg(CGC)	A	A	G	G	2.95×10^{-4} (BW)
		mCV22789398	His(CAT)291Leu(CTT)	A	A	T	T	
Zc3h7a(A430104C18Rik)	mCG126558	mCV22788643	His(CAT)512Arg(CGT)	A	A	G	G	
		mCV22788616	Thr(ACT)371Ala(GCT)	G	G	A	A	
LOC622271	mCG126560	mCV25081620	Arg(AGG)61Trp(TGG)	A	A	T	T	
Mkl2 (Mrtf-B)	mCG123888	mCV24574661	Asn(AAC)369Ser(AGC)	A	A	G	G	8.75×10^{-3} (BG)
		mCV24574662	Ser(AGC)377Gly(GGC)	G	G	A	A	2.69×10^{-4} (insulin)
		mCV24574663	Ser(AGC)379Gly(GGC)	A	A	G	G	2.69×10^{-4} (diabetes)
		mCV24574670	Thr(ACC)447Ala(GCC)	A	A	G	G	
3110001I22Rik	mCG129801	mCV25192174	Gln(CAG)286Arg(CGG)	G	G	A	A	
2210010A19Rik (AA415817)	mCG129810	mCV24860060	His(CAC)423Gln(CAG)	C	C	G	G	
D16Mit144								

Genes in boldface denote the most probable candidate gene by the PosMed database.
[a] *p* value denotes the level of association for phenotypes in parentheses.
[b] All mis-sense mutations are nonconservative amino acid changes.

advantages include minimal environmental variability and genetic homogeneity. In this study, we analyzed F_2 populations of F_2-*db/db* and F_2-*db/−* to identify the susceptibility QTLs linked to obesity associated with diabetes. We observed large differences in BW (Fig. 2A), fat pad weight (Fig. 2B), and BG concentrations (Table 1) in F_2-*db/db* and F_2-*db/−* compared with BKS-*db/db* and BKS-*db/−*, respectively. Almost all T2D-related phenotypes were aggravated in male and female F_2-*db/db* and male and female F_2-*db/−* compared to BKS-*db/db* and BKS-*db/−*, respectively. The aggravation of diabetes-related traits in these F_2 subpopulations is consistent with our previous QTL study on a cross of a BKS-*db/−* with DBA [25]. These results indicate that the severity of diabetes-related phenotypes is strongly dependent on the genetic background of C3H or DBA. The main QTL that we detected on Chr 9 in female F_2-*db/db* subpopulation in our previous study, however, was not detected in female F_2-*db/db* subpopulation in the current study on a cross of BKS-*db/−* with C3H. This suggests that QTL on Chr 9 in female F_2-*db/db* subpopulation is specific to DBA.

In this study, we examined four subpopulations in the F_2 population divided by sex and *db* genotype. These subpopulations should have excluded false QTLs by standardization of the structure of the F_2 populations. We observed subpopulation-dependent variable locations of QTLs that increased BW, fat pad weight, and BG concentrations.

We detected three major QTLs including one QTL on Chr 3 in male F_2-*db/db* and two QTLs on Chr 15 and 16 in male F_2-*db/−* in four subpopulations. In male F_2-*db/db*, one QTL on Chr 3 was specific for fat pad weight and not significantly linked to BW, suggesting that the gene(s) in this QTL controlling fat pad weight are different from those controlling BW. For this QTL on Chr 3, CC mice exhibited lower fat pad weight than BB mice (Table 3). However, F_2-*db/db* exhibited higher fat pad weight than BKS-*db/db* (BB homozygous) (Table 1). The phenotype of increased fat pad weight in F_2-*db/db* as a whole is in a sharp contrast with the decreased fat pad weight in F_2-*db/db* with CC genotype of the QTL at *D3Mit86*. This suggests that the phenotype in F_2-*db/db* as a whole with respect to fat pad weight is supposed to reflect the sum of the contributions of many loci with multiplicative or additive interactions. The susceptibility gene that controls fat pad weight at the *D3Mit86* QTL has the potential to provide a new target to control the metabolic syndrome including diabetes, obesity, and intestinal fat deposits.

In male F_2-*db/−*, one QTL on Chr 16 at *D16Mit81* showed the strongest and highest linkage to BG concentrations at 30, 60, and 120 min during the *ip*GTT but not to fasting BG concentrations at 0 min. This finding suggests that different mechanisms regulate fasting and nonfasting BG concentrations in response to glucose administration. Another QTL at *D15Mit107* on Chr 15 was specifically linked to BW from 6 to 11 weeks of age. For these two main QTLs, CC mice exhibited higher BW and BG concentrations at 30, 60, and 120 min in the *ip*GTT than BB mice (Table 3). CC mice at *D15Mit107* also exhibited higher fat pad weight than BB mice. As shown in Table 1, F_2-*db/−* and F_2-*db/db* exhibited higher levels for most phenotypes including BW, fat pad weight, and BG concentrations in the *ip*GTT than BKS-*db/−* and BKS-*db/db*, respectively. Based on these observations, we propose that the C3H genetic background carries genetic factors which aggravate diabetes and obesity.

We analyzed the genome scan in two F_2 subpopulations, i.e., F_2-*db/db* which exhibits diabetic phenotypes and F_2-*db/−* which does not develop diabetes. Detection of three QTLs in our study was shown to be F_2 subpopulation specific. Thus, the observation that the two strongest QTLs contribute to BW and BG concentrations only in F_2-*db/−* suggests that the gene(s) in these QTLs modify these phenotypes under nondiabetic conditions associated with the genotype of *db/−*.

It is important to note that these three QTLs were observed in only one subpopulation of male or female, F_2-*db/db* or F_2-*db/−*. In contrast, no QTLs detected in F_2-*db/db* overlapped with those detected in F_2-*db/−*. The QTLs varied with sex and *db*-dependent diabetes severity. Most of the identified QTLs showed their effects specifically in male or female populations in the presence or absence of *db*-induced diabetes. Incorporating covariates into genome scans indicated highly conditional effects of the QTLs which we attribute to two causes. First, different QTLs were detected for various T2D-related phenotypes in the presence or absence of *db*-induced obesity. Second, the majority of QTLs were sex dependent, having been found predominantly in male mice. These observations imply that susceptibility genes in the QTLs interact with clinical factors such as obesity or nonobesity. Significance linkage threshold values were consistent with the threshold values in permutation tests. On the basis of these tests, we selected three QTLs as the major susceptibility QTLs for T2D-related phenotypes. Interestingly, the QTL on Chr 5 between *D5Mit356* and *D5Mit135* for BW was observed commonly in both male and female F_2-*db/db*, despite the relatively low LOD scores. This region coincided with the QTL reported in New Zealand obese mice fed with a high fat diet [26].

While many QTLs have been reported, only a few have been successfully described at the molecular level [27]. This is likely due to the difficulties encountered in mapping responsible genes in QTLs and the effects of strain background [28]. The ultimate goal of a susceptibility QTL study is to identify the gene(s) that change phenotypes. Recently, genome-wide SNP data and defined haplotype structure across the genome of different mouse strains have become available, enabling their application to the fine mapping of QTLs [23,29,30]. Homozygosity at all loci in inbred strains has important implications for mapping QTL regions in intercross progeny with easily discernible haplotypes because shared haplotype blocks reduce the region of the genome to be queried for biological variation. Identification of polymorphic variants based on the SNPs database helped us to define the haplotype structures between the BKS and the C3H strains. The reduction in our target region from 20 Mb between the telomere and 19,999,970 to 4.6 Mb in size should accelerate the identification of susceptibility gene(s).

Amino acid substitutions are also important in providing functional variations. Genes that have cSNPs accompanied with amino acid variations select a group of candidate genes in the target region. The basis of each QTL is a sequence variant that defines a difference in gene expression or function contributing to phenotypes. We identified 11 nonsynonymous SNPs in six candidate genes and identified two candidate genes of *Txndc11* (thioredoxin domain containing 11: thioredoxin-related protein) and *Mkl2* (MKL/myocardin-like 2: transcriptional coactivator) genes, which are associated in the database with BG concentrations, insulin, and T2D-related phenotypes. These genes were considered the candidates.

We conclude that a genome-wide scan of the F_2 population divided by sex and *db* genotype identified three major QTLs: one on Chr 3 for fat pad weight, one on Chr 15 for BW, and one on Chr 16 for BG concentrations. For the most highly significant QTL on Chr 16, we identified 11 nonsynonymous cSNPs in six candidate genes by our combined approach based on the comparison of detailed haplotype structures of the strains and interstrain sequence variations.

Materials and methods

Mice and F_2 populations

Parental inbred male and female strains included BKS.Cg-+*Lepr*^{db}/+*Lepr*^{db} (BKS-*db/db*), BKS.Cg-*m*+/+*Lepr* (BKS-*db/*−), BKS.Cg-*m*+/*m*+ (BKS- -/-), and C3H. They were purchased from CLEA Japan Inc. (Tokyo, Japan) for phenotypic comparisons with F_2 populations. Male BKS-*db/*− was crossed with female C3H to generate F_1 mice because homozygotes for the *db* mutation are sterile. F_1 mice heterozygous for *db* were then intercrossed to generate the F_2 population. We obtained 785 F_2 mice including F_2-*db/db* (n=227), F_2-*db/*− (n=378), and F_2- -/- without the *db* mutation (n=180). In this study, F_2- -/- were not analyzed because we focused on genotypes for *db*.

In our 1st step analysis of linkage, F_2-*db/db* and F_2-*db/*− were separated by sex and *db* genotype to map the responsible QTLs for their conditional effects. We used male F_2-*db/db* (n=118), female F_2-*db/db* (n=109), male F_2-*db/*− (n=168), and female F_2-*db/*− (n=210), separately. In our 2nd step analysis of linkage, we analyzed the sex-dependent effects of the QTLs using combined populations of F_2-*db/db* (n=227) or F_2-*db/*− (n=378) with sex. In our final study of linkage, we analyzed sex-, *db*-, and sex-by-*db*-dependent effects of QTLs using the whole combined population of F_2-*db/db* and F_2-*db/*− with sex (n=605) (Fig. 1).

All pups were weaned at 4 weeks of age (29±1 days) and were then fed ad libitum with standard laboratory chow (MF, Oriental Yeast Corp.) and maintained under a 12-h light and dark cycle (8 am/8 pm). The experimental protocol for animal use was approved by the Animal Care Committee of the University of Tokushima.

Genetic diagnosis of the db mutation in F_2 populations

We extracted genomic DNA from the tail tips of all F_2 progenies and their parent F_1 mice at 4 weeks of age using DNeasy 96 tissue kit (Qiagen, Hilden, Germany). The genotype at the *db* locus was determined by the TaqMan-PCR method (Applied Biosystems (ABI), Foster City, CA). The minor groove binder (MGB) TaqMan probe was created using a G/T point mutation of the leptin receptor [12,31]. The sense primer used was 5′-CAACTTCCCAACAGTCCATACAATATTA-3′, while the antisense primer was 5′-AAACTGAACTACATCAAACCTACATTGTG-3′. TaqMan MGB probes used were 5′-FAM-TGGAGGGAAACAAA-MGB-3′ for the wild type and 5′-VIC-TGGAGGTAAACAAAC-MGB-3′ for the mutation type. The PCR assay was based on the manufacturer's recommendation.

Time schedule of phenotypic trait measurement

In F_2 populations, F_2-*db/db* or F_2-*db/*− were sacrificed at 9 weeks of age (63±1 days) or 11 weeks of age (77±2 days), respectively. The *ip*GTT was performed at 8 weeks of age for F_2-*db/db* (56±1 days) and at 10 weeks of age for F_2-*db/*− (70±2 days) after fasting for 16 h. We measured 20 phenotypes for F_2-*db/db* and 23 phenotypes for F_2-*db/*− (Supplementary Table 1) as defined by the following parameters: (1) nonfasting BW was serially measured every week from weaning until sacrifice, and fasting and nonfasting BW was measured at sacrifice; (2) fasting BG concentrations were measured once every week and at sacrifice, nonfasting BG concentrations were measured before the *ip*GTT, and BG concentrations were measured during the *ip*GTT at 0, 30, 60, and 120 min; (3) abdominal fat pad weight (epididymal in male and parametrial in female) was measured at sacrifice; and (4) serum concentrations of insulin were measured at 30 min after glucose injection and at sacrifice, and serum concentrations of leptin and TG were measured at sacrifice. BG concentrations were monitored using a glucose analyzer (Antsence II, Bayer Sankyo Inc., Japan). The *ip*GTTs were performed by injecting 2 mg/g BW of glucose in physiological saline after mice had fasted for 16 h. Blood samples were collected from the abdominal aorta under ether anesthesia, followed by the collection of fat pads and measurement of their weight.

Genotyping by microsatellite markers

We selected a set of 203 well-validated microsatellite markers (ABI) which differed by 2 bp or more in the BKS and C3H strains. Information on these polymorphisms was obtained from the mouse genome database at the Whitehead Institute/MIT Center (http://www.genome.wi.mit.edu/cgi-bin/mouse/index) and from an online database of the Jackson Laboratory Center (http://www.informatics.jax.org/).

All 203 microsatellite markers were genotyped in the entire F_2 population in a reaction volume of 5 μl that included 4.6 μl of the reaction mixture and 0.4 μl of DNA solution with the fluorescently labeled sense primer including HEX, FAM, or NED. The PCR amplicons were electrophoretically separated on a 3700 capillary DNA sequencer (ABI). Two researchers independently performed the size determination for each marker in the two strains using Genotyper 3.7 (ABI).

Statistical analysis of phenotypic data

Statistical analyses were carried out using Statview 5.0 (SAS Institute Inc., Cary, NC, USA). The distribution of BW data was examined using box (median value±one quartile) and whisker plot above and below one quartile with the bars representing 10 and 90 percentiles. To evaluate the effects of genotype of the QTLs, F_2 subpopulation was separated according to the genotype of the nearest marker of the QTLs into BB (BKS homozygous), BC (heterozygous), and CC (C3H homozygous). Phenotypic comparisons in different genotype groups were performed by the classical Kruskal–Wallis test with Scheffe's test being used for post hoc multiple comparisons.

Genome-wide QTL genome scans

We carried out a separate genome scan in each of four subpopulations defined by sex or *db* genotype. Genome-wide scans were implemented by R/qtl (http://www.biostat.jhsph.edu/~kbroman/qtl/) with Haley–Knott regression [32]. Haley–Knott regression required multipoint genotype probabilities and were calculated at 2 cM intervals for the maximal distance between positions.

Covariate-dependent genome scans

The covariate-dependent genome scan has been described in detail [33]. A simple genome scan compares two linear models written as

$$y_i = \beta_0 + \varepsilon_i \text{ and} \quad (1)$$

$$y_i = \beta_0 + \beta_1 Q_i + \varepsilon_i, \quad (2)$$

where y_i is the phenotype, β_0 and β_1 are regression coefficients, Q represents the QTL genotype, and ε_i are normal errors. The index i runs through all individuals in the cross. The LOD score is the difference in $\log_{10}$ likelihood values in models (1) and (2), where the individual model likelihoods are maximized with respect to the regression coefficients. If a phenotype differs on average in the two sexes but the QTL has the same effect on both males and females, this can be modeled by including sex as an additive covariate in the genome scan. The genome scan with additive covariates provides LOD scores contrasting the models

$$y_i = \beta_0 + \beta_1 X_i + \varepsilon_i \text{ and} \tag{3}$$

$$y_i = \beta_0 + \beta_i X_i + \beta_2 Q_i + \varepsilon_i, \tag{4}$$

where X is a covariate. A genome scan based on the LOD score contrasting models (3) and (4) accounts for the effects of the covariate. Alternatively, if a QTL has an effect in only one of the sexes, we can allow for covariate-dependent QTL effects by using a linear model that includes a QTL-by-covariate interaction term:

$$y_i = \beta_0 + \beta_1 X_i + \beta_1 X_i + \beta_2 Q_i + \beta_3 Q_i X_i + \varepsilon_i. \tag{5}$$

To make inferences about covariate-dependent QTL effects, one must consider all three models (3), (4), and (5).

Using sex as an additive covariate {model (4)–model (3)}, we reanalyzed the traits that showed the highest LOD score in the genome-wide scan in subpopulations. For this analysis, the subpopulations of F_2 mice were combined with sex but divided by *db* genotype. They were also reexamined by using sex as an interactive covariate {model (5)–model (3)}. The change in likelihood between models (4) and (5) at the peak position helped us to identify sex-specific effects of the QTLs. Significance thresholds of covariate-dependent genome scans ($\alpha = 0.05$) were also generated in R/qtl by permutation tests ($n = 500$ permutations) [20,22]. In the whole combined population, we could merge the data that were collected at the same time points in F_2.*db*/*db* and F_2.*db*/− populations. We also ran additional genome scans with sex, *db* genotype, and sex-by-*db* genotype, respectively, as additive and interactive covariates.

Discovery and validation of Celera SNP and detection of interstrain polymorphisms in silico

We selected SNPs accompanied by polymorphisms in silico in B6 and DBA mice in our target region of 20 Mb based on the Celera database. All SNP sequences within the target region were downloaded from the Celera Mouse Genome Assembly under the Celera Discovery System (http://www.celera.com). The Celera Mouse RefSNP database (release 1.0) was constructed by using data collected at Celera from strains 129x1/SvJ, 129S1/SvImJ, D2J, and B6 mice strains containing 2,566,706 SNPs. This database was utilized to screen for SNPs that cause polymorphisms and cause mis-sense mutations between B6 and DBA within the target region. These predicted polymorphisms were confirmed to be polymorphic between BKS and C3H by sequencing the BKS and C3H genomes at 50-kb intervals within the target region. Selected SNPs that cause mis-sense mutations between B6 and DBA were confirmed to be polymorphic between BKS and C3H by sequencing.

Direct sequene analysis of BKS genome to confirm SNPs

Oligonucleotides were designed to identify the genome sequence between BKS and C3H strains of which SNP sequences were not available from the Celera database. Ten-microliter PCRs consisted of 8.0 μl PCR Master Mix containing 0.02 U/μl KOD plus (Toyobo, Japan), 1.0 μl of 50-ng/μl DNA, 1 μl of 1 μM forward, and reverse primer. All PCRs were performed with the following cycling conditions: 94 °C for 2 min followed by 30 cycles of 94 °C for 15 s., 56 °C for 30 s, and 68 °C for 30 s. PCR amplicons were directly sequenced from both strands with BigDye Terminator Cycle Sequencing-Ready Reaction Kit (v3.1 or v1.1 ABI). One microliter was genotyped by electrophoresing the fluorescence-labeled PCR amplicons with an ABI Prism 3730xL DNA Analyzer followed by analysis using GeneMapper (v3.0).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2006.07.002.

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