# Genetic Obesity Unmasks Nonlinear Interactions Between Murine Type 2 Diabetes Susceptibility Loci

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**Nonlinear interactions between obesity and genetic risk factors are thought to determine susceptibility to type 2 diabetes. We used genetic obesity as a tool to uncover latent differences in diabetes susceptibility between two mouse strains, C57BL/6J (B6) and BTBR. Although both BTBR and B6 lean mice are euglycemic and glucose tolerant, lean BTBR × B6 F1 male mice are profoundly insulin resistant. We hypothesized that the genetic determinants of the insulin resistance syndrome might also predispose genetically obese mice to severe diabetes. Introgressing the *ob* allele into BTBR revealed large differences in diabetes susceptibility between the strain backgrounds. In a population of F2-*ob/ob* mice segregating for *BTBR* and *B6* alleles, we observed large variation in pancreatic compensation for the underlying insulin resistance. We also detected two loci that substantially modify diabetes severity, and a third locus that strongly links to fasting plasma insulin levels. Amplification of the genetic signal from these latent diabetes susceptibility alleles in F2-*ob/ob* mice permitted discovery of an interaction between the two loci that substantially increased the risk of severe type 2 diabetes. *Diabetes* 49:1946–1954, 2000**

Type 2 diabetes involves insulin resistance coupled with failure of the pancreatic β-cells to secrete enough insulin to maintain euglycemia (1–3). Although insulin resistance is a feature of type 2 diabetes, an individual can be severely insulin resistant without ever exhibiting fasting hyperglycemia; β-cell insufficiency is an essential feature of type 2 diabetes (4).

Obesity is an important independent risk factor for the development of type 2 diabetes: more than 80% of type 2 diabetic patients are obese. Nevertheless, although most obese people are insulin resistant, the majority remains euglycemic. There are few biochemical clues that allow us to predict which obese individuals will progress to type 2 diabetes, much less why they progress. In addition, there is limited mechanistic information to help us understand why obesity is so intimately related to diabetes.

A strong genetic contribution to type 2 diabetes is suggested by the clustering of this trait within families and ethnic groups, and by its high concordance (50–95%) between monozygotic twins (5). Mutations at many loci (e.g., insulin receptor, glucagon receptor, insulin, *GLUT1*, *GLUT4*, and insulin receptor substrates [IRS] –1 and –2) have been shown to cause single-gene or oligogenic diabetes syndromes, but these mutations together account for <1% of all human cases of type 2 diabetes (6). Approximately 1–3% of diabetes cases are inherited in a dominant form called maturity-onset diabetes of the young (MODY). Five MODY genes have been positionally cloned. Despite these advances, the identities of the major type 2 diabetes susceptibility genes remain unknown.

There is a growing consensus that for the vast majority of cases of type 2 diabetes, multiple genes synergistically influence disease susceptibility (5,6). Oligogenic diabetes syndromes have been modeled in transgenic mice; mice heterozygous for null alleles at the insulin receptor and the *Irs1* genes develop diabetes, whereas mice with just one of these mutations are not diabetic (8). This model illustrates that reduction in function at single loci may not be sufficient to raise an individual's risk of diabetes; interactions between susceptibility loci can extensively modify the overall risk.

The interaction between obesity and genetic type 2 diabetes risk has been studied extensively in the Pima Indians of Arizona and northern Mexico (9). In the early 20th century, the prevalence of type 2 diabetes in Pima Indians was no higher than that in the overall U.S. population (10). Today, the prevalence of type 2 diabetes approaches 50% in Pima adults within the Gila River Community in Arizona. This increase coincides with fundamental changes in diet and lifestyle. Prevalence of type 2 diabetes is low in Pima subpopulations in northern Mexico who live a more traditional lifestyle and have lower BMI, despite presumably sharing some or all of the diabetogenic alleles with their Arizona counterparts (11). These studies indicate that effects of diabetogenic alleles may be latent or undetectable in lean individuals. Thus, an interaction between BMI and parental diabetes status has long been known (12), but the genetic or biochemical basis for this interaction has yet to be established.

In laboratory animals, diabetogenic alleles have been modeled as modifier genes, whose effects alter the phenotype of animals genetically predisposed to obesity. Some of these models have used null alleles of the leptin gene (such as *ob*) or its receptor (such as *db* or *fa*) as backgrounds upon which segregating modifier genes can be studied. The studies of Coleman and

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ANOVA, analysis of variance; CIM, composite interval mapping; IRS, insulin receptor substrate; LOD, logarithm of odds; MODY, maturity-onset diabetes of the young; NHLBI, National Heart, Lung, and Blood Institute; PBS, phosphate-buffered saline; QTL, quantitative trait loci; RIA, radioimmunoassay.

Hummel (13) first showed that the phenotype of *ob/ob* mice is modified extensively by strain differences. C57BL/6J-*ob/ob* mice develop modest transient hyperglycemia, but C57BL/KsJ-*ob/ob* mice develop severe progressive hyperglycemia. Loci that extensively modify body mass and fasting insulin levels in *fa/fa* rats have also been reported (14).

We have previously described a novel model of insulin resistance in mice (15). When BTBR and C57BL/6J (B6) mice are outcrossed, the F1 males have elevated fasting insulin levels and are profoundly insulin resistant: they have impaired oral glucose tolerance, and attenuated insulin-stimulated glucose uptake into muscle tissue. Such profound insulin resistance is exhibited by neither parental strain, and the effect is apparently specific to progeny of BTBR and B6 mice. Thus, an interaction between previously silent *B6* and *BTBR* alleles appears to elicit the insulin resistance syndrome. Furthermore, parent-of-origin effects do not contribute substantially to the syndrome, because F1 progeny of the reciprocal cross (B6 × BTBR) are not different from BTBR × B6 F1 mice.

To unmask any diabetogenic effects of insulin resistance alleles, we investigated the effect of introducing *ob* into BTBR, which enabled us to demonstrate that unknown alleles within BTBR greatly increase diabetes susceptibility in obese mice. In addition, we produced an F2 population of *ob/ob* mice derived from the B6 and BTBR strains, which enabled us to quantitate the effects of segregating *B6* and *BTBR* alleles to fasting insulin and glucose. Thus, we used obesity to expose the effects of potentially diabetogenic alleles, and to genetically dissect their contribution to diabetes susceptibility.

In the F2-*ob/ob* population, we observed a 7.5-fold range in fasting glucose (100–750 mg/dl) and a 50-fold range in fasting insulin (2.5–125 ng/ml) at 10 weeks. We also uncovered a large variation in islet hyperplasia and pancreatic insulin content, suggesting that proliferative responses of the islet contribute to disease susceptibility. We report the positions of two loci that individually explain a large part of the genetic variation in fasting glucose and/or insulin. Interestingly, an interaction between these two loci extensively modifies risk of severe type 2 diabetes in obese F2 mice.

## RESEARCH DESIGN AND METHODS

**Animals.** BTBR, B6, and B6-*ob*/+ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred at the University of Wisconsin Animal Care Facility. Mice were weaned at 3 weeks of age onto a roughly 6% fat (by mass) diet (Purina #5008). Mice had free access to food and water at all times, except for 4-h fasting periods before blood withdrawal. Ranheim et al. (15) provide a detailed description of the BTBR lineage. The animal facilities and research protocols were approved by the University of Wisconsin–Madison Institutional Animal Care and Use Committee.

**Genotyping.** Genomic DNA was extracted from tail segments. Tail clippings were minced in 50 mmol/l Tris, 100 mmol/l EDTA, 100 mmol/l NaCl, and 1% SDS and digested overnight with 0.3 mg/ml proteinase K. Undigested material was pelleted at 2,000*g* and discarded. DNA was extracted from the supernatant with an equal volume of Tris-saturated phenol, and reextracted with phenol-chloroform. DNA was precipitated with an equal volume of isopropanol, washed with 75% ethanol, and resuspended in Tris-EDTA, pH 8.0.

Genotyping of 160 mice at 99 microsatellite markers (16), spanning the 19 mouse autosomes, was performed using dye-labeled Mouse MapPairs (Research Genetics, Huntsville, AL) by the laboratory of Dr. David Vaske at the National Heart, Lung, and Blood Institute (NHLBI) Mammalian Genotyping Service (Marshfield Clinic, Marshfield, WI).

Additional genotyping for 190 mice was performed in regions showing preliminary linkage to the traits of interest (logarithm of odds [LOD] > 3.0). Polymerase chain reaction was performed according to standard protocols in 96-well plates using a model PTC-100 thermal controller (MJ Research, Watertown, MA). Products were electrophoresed on polyacrylamide gels, stained with SYBR Green I (Molecular Probes, Eugene, OR), and visualized with a Fluorimager SI (Molecular Dynamics, Sunnyvale, CA).

**Phenotypic characterization.** After a 4-h fast from 8:00 A.M. to 12:00 noon, blood was withdrawn from the retro-orbital sinus. Plasma glucose concentration was determined using the glucose oxidase method (Sigma Diagnostics, St. Louis, MO). Plasma insulin concentration was determined by Rat Insulin RIA kit (Linco Research, St. Charles, MO).

Because the variance in insulin values appeared to scale with the mean insulin values for each population and time point, log-transformations were used for genetic analysis of all insulin traits.

The broad-sense heritability of each trait was calculated by subtracting the ratio of the variances of the F1 and F2 populations from unity.

**Pancreatic histology.** Mice were killed by $CO_2$ asphyxiation at 14 weeks of age after a 4-h fast. Pancreases were rapidly dissected and fixed in 10% formalin, embedded in paraffin, and sectioned. Sections were deparaffinized in 100% xylene, serially hydrated, and equilibrated in phosphate-buffered saline (PBS). Endogenous peroxidases were blocked with 0.3% $H_2O_2$ in PBS for 30 min. Sections were incubated with guinea pig anti–pig insulin antiserum (Sigma), rinsed with PBS, and incubated with peroxidase-conjugated anti–guinea pig IgG (Sigma). Immune complexes were visualized with TrueBlue substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and tissue was counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA). Sections were dehydrated into ethanol and mounted.

**Pancreas insulin content.** Whole pancreases were minced and extracted in 0.2 mol/l HCl 75% ethanol for 18 h at –20°C. Insoluble material was pelleted at 2,000*g* for 5 min and discarded. The supernatant was neutralized with 10 mol/l NaOH and diluted 8,000-fold into PBS + 1% bovine serum albumin (radioimmunoassay [RIA] grade; Sigma). Triplicate aliquots were assayed by RIA according to the above-described method.

**Genetic analysis.** Initially, 160 F2-*ob/ob* mice were genotyped at 99 microsatellite markers spanning the 19 autosomes with an average spacing of 16 cM. Within regions of interest, an additional 190 mice were genotyped, and marker spacing was brought to 5 cM. Mapmaker/EXP (17) was used to compile genotype data into a framework map. The maximum-likelihood order was checked against the Chromosome Committee map positions in the Mouse Genome Database (The Jackson Laboratory, Bar Harbor, Maine) (18).

Interval mapping methods implemented in Mapmaker/quantitative trait loci (QTL) (19) and QTL Cartographer (20) were used to compute linkage to the traits of interest, and to investigate mode of inheritance. The traits included plasma glucose and insulin traits measured at 8 and 10 weeks.

For each trait and major locus, the percentage of explained genetic variance is reported. This value was obtained by dividing the total percent variance explained ($r^2$) by the heritability of the trait. This value is not a measure of the correlation between genotype and phenotype, but the proportion of total heritability that is accounted for by the locus.

Multitrait composite interval mapping (CIM) methods implemented in the JZmapqtl algorithm of QTL Cartographer were also used to investigate the linkages (20,21). First, stepwise regression analysis was conducted on the data, using the SRmapqtl algorithm. Markers exhibiting suggestive evidence of linkage were selected for incorporation into a genetic model as background loci. At each test position, linkages to 8- and 10-week traits were jointly assessed after conditioning on these background loci. The LOD scores reported from this method reflect the joint likelihood of linkage to both traits after adjusting for the effects of the background markers.

**Statistics.** Comparisons between groups are performed by analysis of variance (ANOVA), unless otherwise noted.

The difference in plasma glucose linear trend between BTBR-*ob/ob* and B6-*ob/ob* mice was tested using the PROC MIXED procedure in SAS (SAS Institute, Cary, NC) (22). Mice were stratified by sex.

The interaction between *ob*, sex, and strain on 10-week plasma glucose levels was examined by multiple regression (PROC GLM in SAS [22]). Regression terms included sex, strain, *ob* homozygosity, and interactions between them.

A similar multiple regression method, developed by Cockerham (23), was used to investigate interactions between candidate loci on fasting glucose levels in F2-*ob/ob* mice. Regression terms included sex, the additive and dominance effects at each marker locus, and interactions between each additive and dominance term. The full model was reduced by eliminating terms that exhibited poor association with the dependent variable (fasting glucose).

Interlocus interactions on diabetes susceptibility were analyzed by the $\chi^2$ goodness-of-fit test. Male F2-*ob/ob* mice were classified as affected or not affected according to their 10-week fasting plasma glucose (over or under a threshold value of 400 mg/dl). The sensitivity of this method to the threshold value was investigated by varying the threshold and repeating the analysis. From 360 to 500 mg/dl, all threshold values tested yielded highly significant differences between the observed and expected frequencies ($P < 0.001$), indicating that the test was not sensitive to our definition of disease status. Below 300 mg/dl, the expected frequency of unaffected

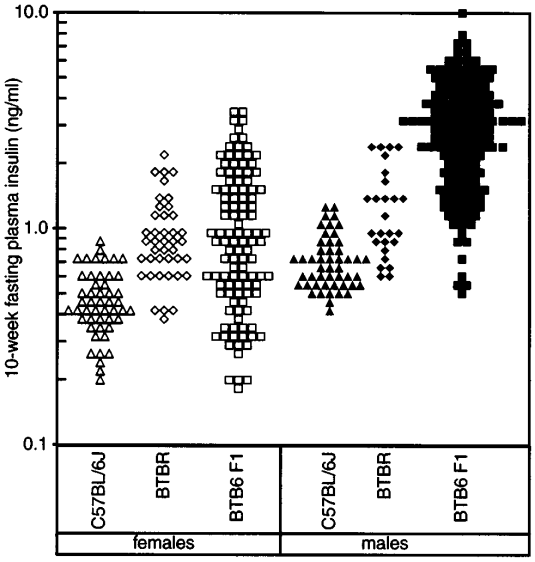


**FIG. 1. Fasting plasma insulin distributions of lean mice by sex and genetic background. Measurements were taken at 10 weeks of age after a 4-h fast. Insulin levels were assayed by RIA. Mean fasting insulin levels for F1 progeny of the reciprocal cross (B6 × BTBR) are not different from BTBR × B6 F1 mice ($P > 0.05$, $t$ test, data not shown).**

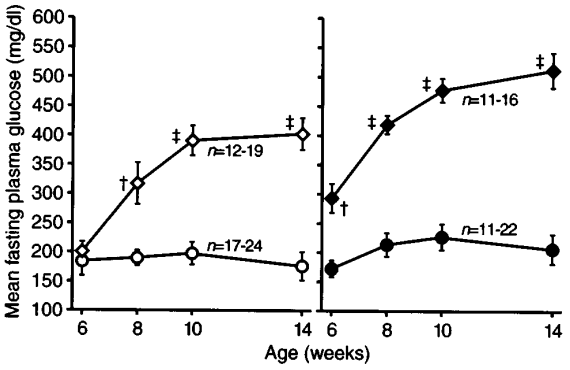


**FIG. 2. Fasting plasma glucose of *ob/ob* mice by sex and genetic background, from 6 to 14 weeks of age. Blood was collected after a 4-h fast. Glucose levels were assayed by the glucose oxidase method. Mean ± SE values for each time point are shown. ◇, BTBR-*ob/ob* females; ○, B6-*ob/ob* females; ◆, BTBR-*ob/ob* males; ●, B6-*ob/ob* males. Strain differences at each time point are significant at †$P < 0.05$ and ‡$P < 0.001$. Furthermore, BTBR-*ob/ob* and B6-*ob/ob* mice show highly significant differences in plasma glucose linear trend ($P < 0.0001$ in both males and females).**

individuals in each haplotype approaches zero, and the $\chi^2$ test becomes unreliable. At a threshold of 200 mg/dl, virtually all males are affected, regardless of genotype.

## RESULTS

**Insulin resistance and fasting hyperinsulinemia in lean mice.** We have previously shown that although the B6 and BTBR mouse strains are glucose tolerant, the BTBR × B6 (BTB6) F1 males are profoundly insulin resistant (15). Fasting hyperinsulinemia is a marker of insulin resistance due to compensation by the pancreatic β-cells. Accordingly, fasting insulin levels in the F1 males show a highly significant elevation from each parental strain ($P < 0.0001$, F1 vs. BTBR and F1 vs. B6; Fig. 1). Previously, we reported increases in body weight and visceral fat mass that paralleled fasting insulin levels in F1 male mice (15). Sex dimorphism of diabetogenic traits has been observed in many rodent models (14,24,25) and was observed throughout our study as well.

Because the whole-animal insulin resistance of the F1 animals is not a trait of either parental strain, alleles from both parental strains might be required for the insulin resistance of the F1 animals. Thus, a satisfactory genetic explanation of the F1 phenotype must recognize the potential existence of deleterious alleles within B6 as well as BTBR.

**The *ob* allele unmasks diabetes alleles in BTBR mice.** We hypothesized that any allele promoting insulin resistance in lean mice might promote progression to type 2 diabetes in genetically obese mice. We therefore introgressed the *ob* allele from B6 into BTBR using a marker-assisted backcrossing program (26) for six generations. After six generations of backcrossing, using 60 markers (3 per chromosome), we encountered no *B6* alleles anywhere other than on chromosome 6, in a small interval flanking the *Lep* locus, indicating that N6 backcross mice are essentially congenic for the *ob* allele. The N6-*ob*/+ congenic mice were intercrossed to produce *ob/ob* mice with a BTBR background (BTBR-*ob/ob*). Crossing N6-*ob*/+ congenic mice to B6-*ob*/+ produced *ob/ob* mice on the BTB6 F1 hybrid background (BTB6-*ob/ob*). We also generated a panel of 350 BTBR × B6 F2-*ob/ob* mice by crossing BTBR-+/+ with B6-*ob*/+, and intercrossing the F1-*ob*/+ progeny. In short, we generated *ob/ob* mice on the BTBR, B6, F1, and F2 genetic backgrounds, allowing us to dissect the effects of diabetes susceptibility alleles present within the parental genetic backgrounds.

Introgression of *ob* into the BTBR strain produced dramatic hyperglycemia relative to B6-*ob/ob* mice (Fig. 2). The strain difference is significant by ANOVA starting at 8 weeks of age. The hyperglycemia in B6-*ob/ob* mice partially remits between 10 and 14 weeks of age, consistent with the findings of several laboratories (13,27). However, fasting glucose levels in BTBR-*ob/ob* mice continue to rise through the first 14 weeks of life. Therefore, in contrast to the mild transient hyperglycemia typical of B6-*ob/ob* mice, the diabetes syndrome exhibited by BTBR-*ob/ob* mice is severe and progressive.

Infrequently, we observed B6-*ob/ob* mice that transition to severe hyperglycemia (Fig. 3). Phenodeviant mice such as these have been previously described (27). Currently, there is no satisfactory explanation for the existence of these phenodeviants, but the results do not prove that the B6 strain is genetically heterogeneous. Rather, the results may simply suggest that B6 bears low-penetrance alleles that promote a transition to severe diabetes, and that environmental variation leads to the decompensation. A satisfactory genetic dissection of diabetes in B6-*ob/ob* mice might explain the existence of these exceptional mice.

If the transition to severe diabetes were a stochastic process based on variably penetrant susceptibility alleles, then we could examine the effects of strain background in terms of disease susceptibility, rather than as differences between mean fasting glucose levels (Fig. 3). Two threshold glucose levels—250 and 400 mg/dl—illustrate this strain difference in females and males, respectively. Using these threshold values as indicators of relative disease severity, we see that the BTBR background is associated with great increases in susceptibility to severe hyperglycemia (odds ratio 8.3 and 5.2 in male and female *ob/ob* mice, respectively).

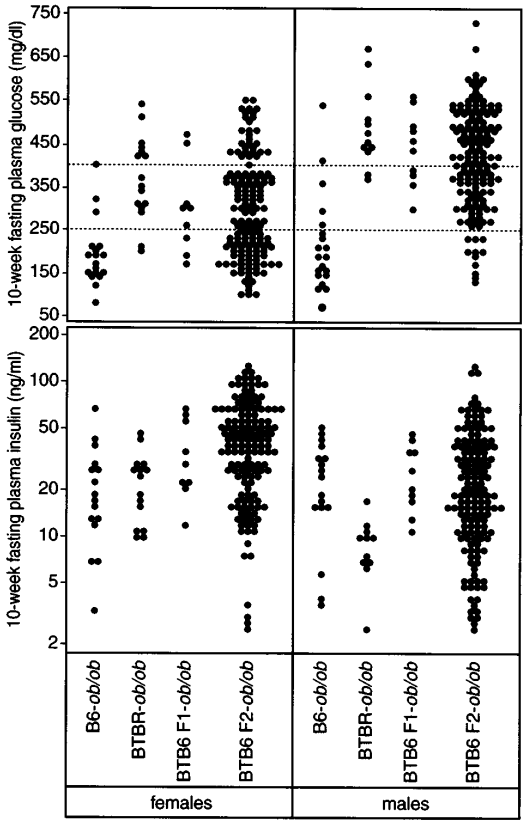


**FIG. 3. Fasting plasma glucose and insulin distributions of *ob/ob* mice by sex and genetic background. Blood was collected at 10-weeks of age after a 4-h fast.**

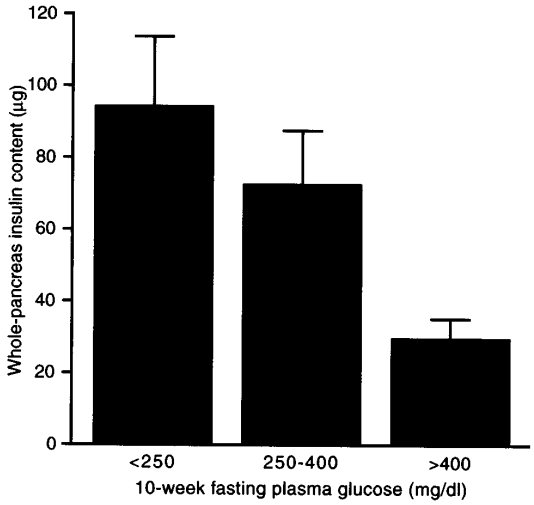


**FIG. 4. Whole-pancreas insulin content of 14-week-old F2-*ob/ob* mice. Animals were grouped by fasting plasma glucose measured at time of sacrifice. Mean ± SE insulin content is shown. Masses of the pancreases are invariant between the groups (data not shown).**

An interaction between *ob* and putative diabetes susceptibility alleles accounts for most of the difference in fasting glucose between BTBR-*ob/ob* and B6-*ob/ob* mice. Whereas the difference in 10-week fasting glucose between lean B6 and BTBR males is undetectable (135 vs. 131 mg/dl, $P > 0.05$), there is a highly significant difference between B6-*ob/ob* and BTBR-*ob/ob* males ($P < 0.0001$). The statistical interaction between strain and *ob* homozygosity, by multiple regression, is large and highly significant ($F > 140$, $P < 0.0001$). Formally, the *Lep* locus is epistatic to diabetes susceptibility alleles in BTBR and B6.

**Loss of β-cell mass in decompensated F2 mice.** The extraordinarily wide variation in fasting insulin levels in F2-*ob/ob* mice could be due to variation in the degree of insulin resistance and/or to differences in β-cell compensation. To investigate compensation, we measured insulin content of whole pancreases from F2-*ob/ob* mice. If insulin resistance were the sole determinant of diabetes severity in F2 mice, then whole-pancreas insulin content should be invariant, or be positively correlated with fasting glucose levels. Instead, pancreases from mildly diabetic F2-*ob/ob* mice (glucose <250 mg/dl) contain significantly more insulin than those from moderately diabetic and severely diabetic mice (Fig. 4; $P < 0.002$, ANOVA), but whole-pancreas masses are invariant (data not shown).

Low whole-pancreas insulin content in severely diabetic mice may reflect pathologic changes in β-cell mass, or may simply reflect lower steady-state insulin content per cell, because of increased secretion rate. We examined sectioned pancreases from F2-*ob/ob* mice by immunostaining with anti-insulin antibodies (Fig. 5). Pancreases from mildly diabetic F2-*ob/ob* mice contain a large number of hyperplastic islets. In striking contrast, islets from moderately and severely diabetic mice show no such hyperplasia but exhibit increasing cellular disorganization and fibrosis. Extensive histologic analysis of F2-*ob/ob* mice revealed no evidence of a lymphocytic or inflammatory infiltrate. However, islets from severely diabetic mice exhibit concavities, into which acinar tissue has intruded. We hypothesize that these concavities are the result of reduced islet volume, secondary to apoptosis. Together, immunohistochemical analysis and whole-pancreas insulin content data reveal that reduction in the number of β-cells is a characteristic feature of the severely diabetic F2-*ob/ob* mice.

**Mapping reveals the genetic contribution of each parental strain to the diabetes syndrome.** We conducted a genome-wide search for loci that explain the large strain differences in diabetes-related phenotypes. Genetic analysis of the entire (unselected) F2 population was conducted. We elected not to search for linkage using the methods of DNA pooling (29) or selective genotyping (30,19), because we expected polygenic inheritance, contributions from both parental strains, and perhaps heterosis. Mapping fasting plasma glucose and insulin traits at 10 weeks, we observed strong linkage signals from three principal loci on chromosomes 16, 19, and 2 (Fig. 6), which we designate type 2 diabetes mellitus, *t2dm1*, *t2dm2*, and *t2dm3*. For brevity, only 10-week linkage statistics are presented. Linkage results for 8-week traits (not shown) are in qualitative agreement with the 10-week data.

The 10-week fasting plasma glucose trait links to a locus on chromosome 16 (*t2dm1*) with a peak LOD score of 4.4 (Fig. 6). According to the guidelines proposed by Lander and Kruglyak (32), this LOD score represents a significant linkage (genome-wide $P < 0.05$). *T2dm1* accounts for a large part of the strain difference in fasting glucose between BTBR-*ob/ob* and B6-*ob/ob* mice, and for 13% of the genetic variance in the

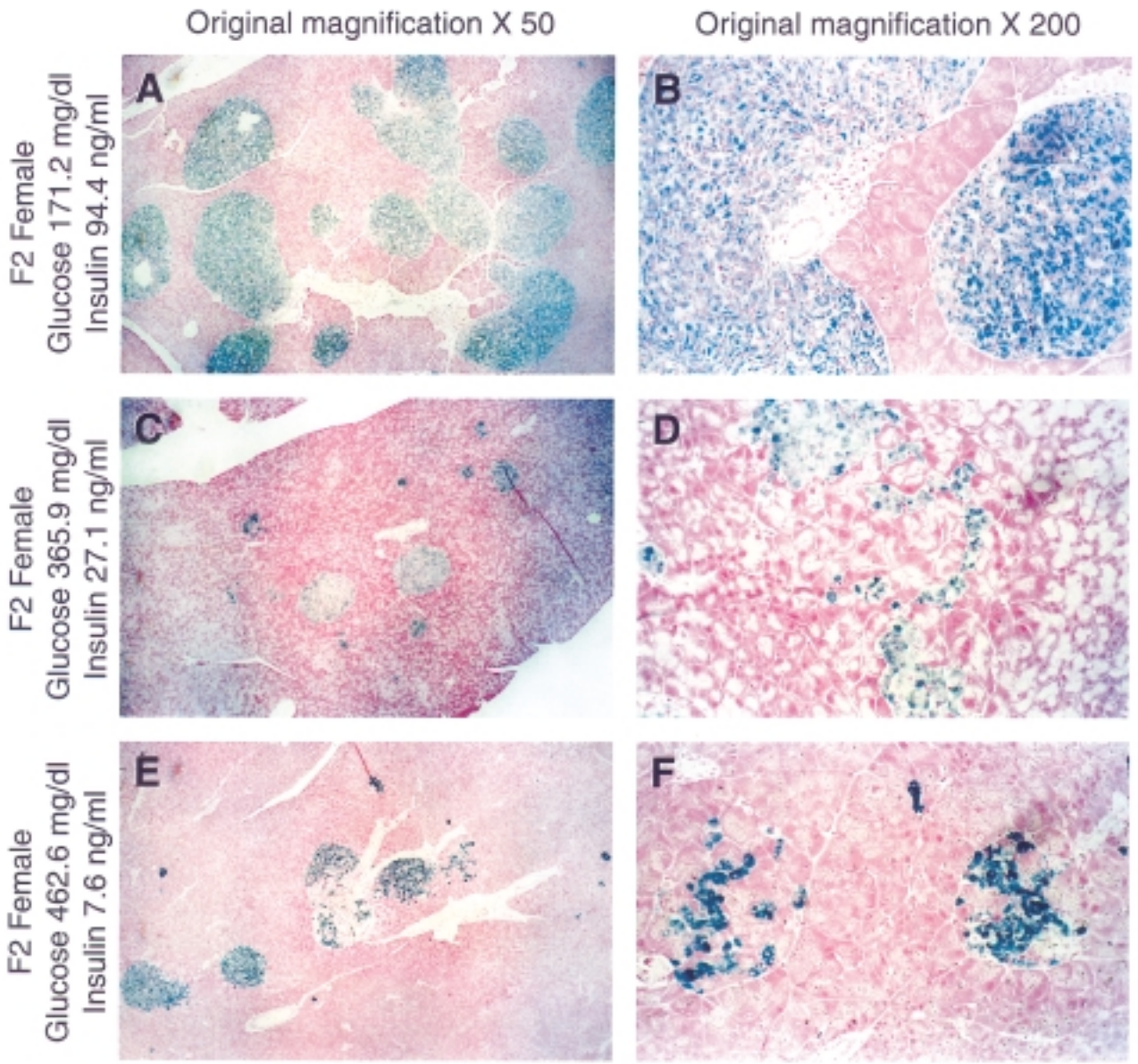


**FIG. 5. Anti-insulin immunohistochemical analysis of pancreases (duodenal end) from F2-*ob/ob* female mice at 14 weeks of age. Representative low- and high-power fields are shown in the left and right columns, respectively. *A* and *B*: Mildly diabetic F2 female exhibits islet hyperplasia. *C* and *D*: Moderately diabetic female exhibits a mixture of large organized islets and clusters of disorganized islet cells with intruding acinar tissue. *E* and *F*: Severely diabetic female shows pathologic changes. Clusters of disorganized islet cells are more frequent. Large islets are poorly organized. Extensive intrusion of acinar tissue into the islets is present.**

F2 population. In male F2-*ob*/*ob* mice, we expect the *B6* and *BTBR* homozygotes at *t2dm1* to differ by ~120 mg/dl of glucose, or approximately half of the total strain difference observed between B6-*ob*/*ob* and BTBR-*ob*/*ob* male mice at 10 weeks. The *BTBR* allele appears to be dominant to the *B6* allele (Table 1), although the degree of dominance varies between fasting glucose and insulin traits.

The 10-week fasting plasma insulin trait shows significant linkage to a locus on chromosome 19 (*t2dm2*) with a peak LOD score of 5.5 (genome-wide $P < 0.01$; Fig. 6), explaining ~18% of the genetic variance in fasting insulin. The expected difference in fasting insulin between *B6* and *BTBR* homozygotes is a factor of 1.91, meaning that each *BTBR* allele is associated with roughly a 38% increase in fasting insulin, and appears dominant (Table 1). *T2dm2* also exhibits suggestive linkage to the fasting plasma glucose trait (peak LOD = 3.79).

BTBR-*ob*/*ob* mice have significantly higher fasting glucose levels than B6-*ob*/*ob* mice. We were therefore surprised to discover that the low-insulin high-glucose allele at *t2dm2* was derived from B6. However, previous work suggests that the B6 strain does indeed carry alleles predisposing to diabetes (25,33); *t2dm2* may harbor one of these. Moreover, these genetic effects potentially explain the existence of rare decompensating B6-*ob*/*ob* mice observed by our group and others (27).

We found a locus on chromosome 2 (*t2dm3*) with significant linkage to the fasting plasma insulin trait (peak LOD = 5.21, genome-wide $P < 0.01$, Fig. 6). This region neighbors the *agouti* and *mahogany* genes, both known to regulate late-

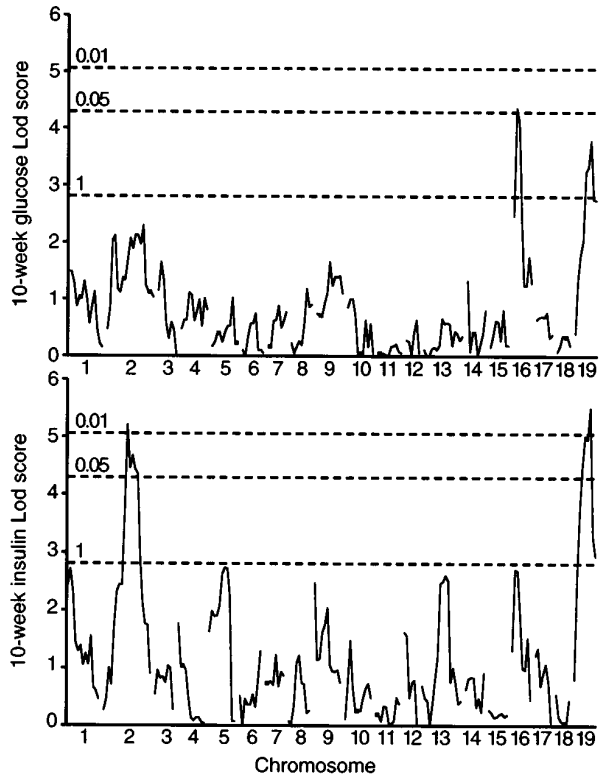


**FIG. 6. Whole-genome interval mapping results for 10-week plasma glucose and insulin traits, summarized by chromosome. Dashed lines indicate genome-wide significance level guidelines. Maximum-likelihood interval mapping techniques were used to generate LOD scores. For brevity, LOD scores for 8-week traits are not shown, but they qualitatively agree with the 10-week results.**

onset obesity/diabetes syndromes in $A^y$ mice (34). The genetics at *t2dm3* best fit an additive genetic model (Table 1).

Two other loci exhibited suggestive linkage to fasting plasma glucose or insulin traits. On chromosome 4, suggestive linkage to 8-week plasma glucose was detected (LOD = 3.0, data not shown). On distal chromosome 5, suggestive linkage to 8- and 10-week insulin was found (LOD = 3.2 and 2.7, respectively; data not shown).

Although the *ob* allele forces body composition in F2-*ob*/*ob* mice toward extreme adiposity, it is possible that small additional variations in body mass (from obesity modifier loci, for example) might modify type 2 diabetes risk. We found two obesity modifier loci (data not shown) in F2-*ob*/*ob* mice, but neither of them exhibit linkage to fasting glucose or insulin levels (conversely, none of the *t2dm* loci shows linkage to body mass). We investigated the connection between adiposity and type 2 diabetes risk by incorporating body mass as a distinct factor in our linkage studies. Using Mapmaker/QTL, we determined that body mass variation within F2-*ob*/*ob* mice shows poor association to fasting glucose levels (LOD < 0.1) and contributes minimally to LOD scores in combination with each *t2dm* locus (incremental LOD < 0.1 after addition of body mass to the model). Thus, we conclude that the small variation in body mass within the F2 population does not explain variation in diabetes risk, rather that the increased risk is most likely conveyed by the large effects of *ob*.

One potential weakness of interval mapping is the occasional detection of false linkages, or ghost QTL, that appear significant because of effects at other loci that co-vary with the test position (either by linkage or coincidence) (35). One way to overcome this problem is to first adjust for genetic effects elsewhere and then compute the linkage statistics at each test position—an algorithm known as CIM (36). If the apparent genetic effects of a given locus are better explained by effects of other loci, then CIM should report a LOD score much lower than that reported by interval mapping algorithms. We used CIM to jointly assess linkage to both 8- and 10- week measurements of plasma glucose and insulin (Fig. 7). We found evidence of linkage between each of the three *t2dm* loci and plasma glucose and/or insulin traits, even after accounting for the effects of all other markers showing suggestive evidence of linkage. These data show that the effects of the *t2dm* loci are not better accounted for by effects of other loci. Furthermore, the position of each LOD peak is in agreement with the position estimated by interval mapping of the 10-week traits.

TABLE 1
Mode-of-inheritance analysis of fasting glucose and insulin traits for the *t2dm* loci

| Locus/Trait | Age (weeks) | $\hat{d}/\hat{a}$* | dom–free† | rec–free‡ | add–free§ | *BTBR* Allele‖ |
|---|---|---|---|---|---|---|
| *t2dm1* | | | | | | |
| Glucose | 8 | 1.02 | –0.05 | –2.37 | –1.30 | Dominant |
| | 10 | 1.18 | –0.00 | –3.74 | –1.64 | Dominant |
| Insulin | 8 | 0.51 | –0.52 | –2.33 | –0.36 | Incomplete dominance |
| | 10 | 0.53 | –0.17 | –1.93 | –0.46 | Incomplete dominance |
| *t2dm2* | | | | | | |
| Glucose | 8 | 0.53 | –0.47 | –0.51 | –0.00 | Incomplete dominance |
| | 10 | 1.21 | –0.02 | –3.26 | –1.13 | Dominant |
| Insulin | 8 | 1.26 | –0.00 | –2.24 | –0.94 | Dominant |
| | 10 | 0.87 | –0.00 | –4.96 | –2.00 | Dominant |
| *t2dm3* | | | | | | |
| Insulin | 8 | 0.73 | –0.11 | –2.70 | –0.73 | Incomplete dominance |
| | 10 | 0.19 | –1.82 | –1.19 | –0.01 | Additive |

*The ratio of dominance ($\hat{d}$) to additivity ($\hat{a}$): $\hat{d}$ is the estimated difference between the mean of the heterozygotes and the mean of the homozygotes, and $\hat{a}$ is the estimated incremental trait change elicited by substitution of one allele for the other. Columns 4–6: reduction in LOD score caused by constraining the free genetic model so that the *BTBR* allele is †dominant, ‡recessive, or §additive. ‖Column 7: summary estimate of the mode of inheritance of the *BTBR* allele.

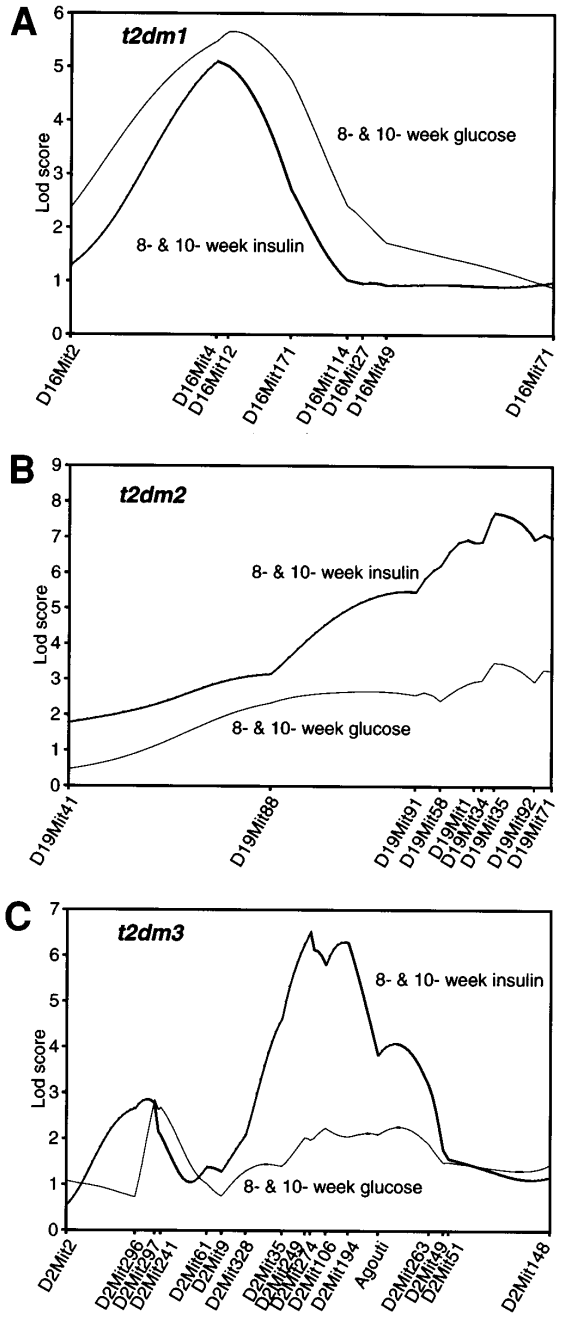


**FIG. 7. Results of multitrait composite interval mapping. Lod scores represent the joint linkage to 8- and 10-week plasma insulin or glucose traits, after accounting for the effects of all markers showing suggestive evidence of linkage, except for markers on the chromosome being studied.**

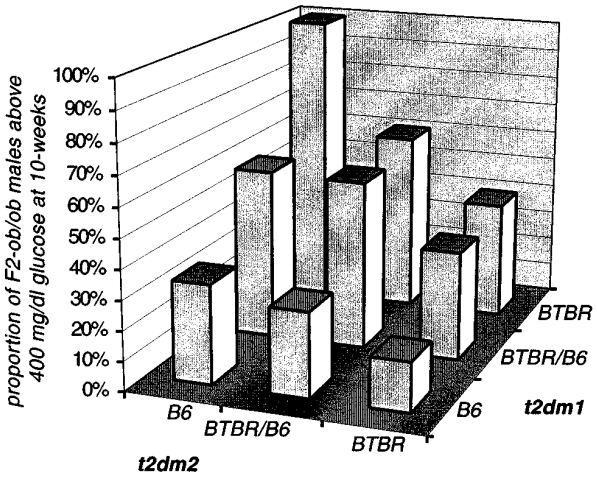


**FIG. 8. In F2-*ob/ob* males, proportion of fasting glucose values >400 mg/dl by genotype at *t2dm1* and *t2dm2*. The statistical interaction between genotypes at *t2dm1* and *t2dm2* is significant, $P < 0.001$, by the $\chi^2$ goodness-of-fit test. The $\chi^2$ test yields $P$ values <0.05 for all thresholds between 350 and 480 mg/dl of glucose.**

We examined the simultaneous effects of *t2dm1* and *t2dm2* on type 2 diabetes susceptibility. If the effects of *t2dm1* and *t2dm2* were independent, we would expect the proportion of decompensating males in each haplotype to be the product of the marginal effects of each locus. We were surprised to find that the observed proportions deviated significantly from this expectation ($P < 0.001$, $\chi^2$ goodness-of-fit test). One particular haplotype, $t2dm1^{BTBR}t2dm2^{B6}$, had an unexpectedly high frequency of decompensating F2-*ob/ob* males (Fig. 8). Of 12 F2-*ob/ob* males identified with this haplotype, all 12 had fasting plasma glucose levels >400 mg/dl. The test was not sensitive to our choice of threshold; results were significant ($P < 0.05$) for all threshold glucose values between 320 and 500 mg/dl, and highly significant ($P < 0.001$) for thresholds between 360 and 500 mg/dl. These data strongly support a nonlinear interaction between *t2dm1* and *t2dm2* that substantially modifies risk of severe diabetes in obese F2 males.

To elucidate the precise nature of the epistasis, we regressed the 10-week glucose trait on markers representing each of the *t2dm* loci, in male F2-*ob/ob* mice. Our analysis followed the method of Cockerham (23): we assessed differences between mean fasting glucose levels, rather than differences in the risk of severe type 2 diabetes. Typical regression models include terms for the additive (or allele substitution) and dominance effects at each locus. We started with a regression model that included interaction terms between each additive and dominance effect, and removed regression terms showing poor association with plasma glucose levels. We found a significant interaction ($P < 0.05$) between the additive genetic effect at *t2dm1* and the dominance at *t2dm2*. This interaction could be interpreted in two ways. First, because the dominance at *t2dm2* appears to increase with each *BTBR* allele at *t2dm1*, *t2dm1* may alter the mode of inheritance of *t2dm2*. Alternatively, because $t2dm2^{B6}$ mice show an especially large additive effect at *t2dm1*, they may represent a class of F2 animals that are especially sensitive to the *t2dm1* genotype.

## DISCUSSION

We have used obesity as a tool to unmask latent diabetogenes present within two mouse strains, B6 and BTBR. Formally, we set up an epistatic interaction between *ob* and diabetes susceptibility alleles present within the parental strains. By doing so, we revealed highly significant differences between the strains, most notably in fasting plasma glucose. The majority of B6-*ob/ob* mice recapitulate the mild transient hyperglycemia originally attributed to the strain (13), but a few progressed to severe diabetes, consistent with

more recent reports (27). In contrast, nearly all BTBR-*ob*/*ob* mice observed to date are severely diabetic. The large and significant strain differences indicate that the *ob* allele amplified the effects of latent diabetogenic alleles present in BTBR. However, the existence of decompensating B6-*ob*/*ob* mice suggests that B6 carries diabetogenic alleles as well, but that the penetrance of these alleles is low in the B6 strain.

We have detected two loci that have large and significant effects on traits directly associated with diabetes severity. The *t2dm1* locus exhibits significant linkage with large effects on fasting plasma glucose. At this locus, the expected male *B6* homozygote differs from the *BTBR* homozygote by 120 mg/dl glucose. Thus, the genetic effects at *t2dm1* explain a large part of the strain difference between BTBR-*ob*/*ob* and B6-*ob*/*ob* mice.

*T2dm1* is syntenic to the long arm of human chromosome 3, and to rat chromosome 11. A neighboring locus, *Dmo9*, was recently found in the OLETF rat, linking to fasting plasma glucose (37). Due to the low positional resolution of *Dmo9*, it is difficult to precisely assess the synteny of *t2dm1* and *Dmo9*.

We found another locus, *t2dm2*, on chromosome 19, with significant linkage to fasting insulin and suggestive linkage to fasting glucose. At this locus, the *B6* allele is associated with the lower plasma insulin and higher plasma glucose. Although the effects at *t2dm2* may seem paradoxical, they may explain the existence of rare decompensating B6-*ob*/*ob* mice and may also explain the genetic contribution of B6 to the BTB6 F1 insulin resistance syndrome predicted by Ranheim et al. (15).

The *t2dm2* locus is approximately syntenic to human 10q24–26. *T2dm2* is also syntenic to a large region on rat chromosome 1 exhibiting linkage to glucose tolerance parameters in the GK × Fisher344 F2 rat (GKF F2), designated *NIDDM1* (38). Other interspecific GK crosses have detected linkage to more centromeric regions of rat chromosome 1 (39), suggesting that a single locus may be present in the GK rat at a position not syntenic to *t2dm2*. Oddly, *NIDDM1* does not link to either fasting plasma insulin or glucose (nominal $P > 0.05$) in the GKF F2 rat, whereas *t2dm2* links strongly to fasting insulin in the BTB6 F2-*ob*/*ob* mouse. For these reasons, the true genetic determinants at *NIDDM1* and *t2dm2* may not be orthologous. Chung et al. (14) detected a locus on proximal rat chromosome 1 showing strong association with pancreatic compensatory response in WKY × 13M F2-*Lepr*$^{fa/fa}$ rats. This locus, however, is not syntenic to *t2dm2*. Later, linkages to "30-minute glucose" were detected in the OLETF rat at a locus near *NIDDM1*, designated *Dmo1* (40).

A third locus, *t2dm3*, is strongly associated with fasting plasma insulin levels. Loci in this region have previously been linked significantly to body fat and fasting insulin levels (41). A neighboring gene, *agouti*, has important effects on obesity and diabetes syndromes in some experimental systems. The agouti protein has been associated with obesity and hyperinsulinemia in $A^y$ mice (34) and with hypersecretion of insulin from cultured human islets (42). BTBR bears the black-and-tan $a^t$ allele of *agouti*, associated with a tan coat color over the ventrum (34,43); this allele is dominant to the nonagouti *a* allele carried by B6 mice. It is unclear whether the $a^t$ allele modifies fasting insulin levels in F2-*ob*/*ob* mice.

Introgression of *ob* revealed unexpected strain differences in pathophysiology within the islets of Langerhans. Severely diabetic F2 mice have low whole-pancreas insulin content and demonstrate a striking loss of islet tissue. In contrast, pancreases from compensating F2 mice exhibit marked hyperplasia of islet tissue and contain relatively large amounts of insulin. Mechanisms that may account for this difference include β-cell neogenesis, proliferation, and/or apoptosis.

β-Cell loss is a feature of both type 1 and type 2 diabetes. In type 1 diabetes, autoimmunity is responsible for β-cell destruction. In type 2 diabetes, β-cell loss is not autoimmunity-mediated, but the precise mechanism is unclear. Islets from decompensating F2-*ob*/*ob* mice are devoid of lymphocytes, granulocytes, and giant cells, indicating that neither autoimmune nor inflammatory processes account for the loss of islet cell mass. These mice, therefore, do not represent a model of type 1 diabetes.

The expected fasting insulin levels of lean B6 and BTBR mice are ~0.7 and 1.3 ng/ml, respectively (Fig. 1). The insulin levels in F2-*ob*/*ob* mice ranged from 2.5 to 150 ng/ml (Fig. 3). Even the most severely diabetic F2-*ob*/*ob* mice are still hyperinsulinemic when compared with their lean siblings. Because these F2 mice secrete insulin at such a high level, they represent a model of type 2 diabetes rather than MODY.

Mechanistically, the interaction between adiposity and the genetic determinants of diabetes are poorly understood. Several investigators have elicited peripheral insulin resistance (25) or diabetes (33) in B6 mice simply by overfeeding with a high-fat diet. Other mouse strains, such A/J, have been more refractory to the development of insulin resistance in response to high dietary fat (33). B6 mice gain more weight than A/J mice without consuming more calories and are also more likely to develop adipocyte hyperplasia (44). These findings imply that B6 may already bear genetic determinants predisposing to the development of insulin resistance and type 2 diabetes. Indeed, here we have discovered a locus at which the *B6* allele is associated with an exacerbation of the diabetes syndrome in F2-*ob*/*ob* mice.

More generally, it is possible that the effects of some human diabetogenes are observable only in the context of an obese individual. Indeed, studies of diabetic Native American populations have revealed that overall genetic type 2 diabetes risk is modified extensively by environmental factors, including obesity. However, this interaction has not been dissected genetically in Pima Indians, and the precise correction for environmental variation in type 2 diabetes susceptibility is not clear.

Leptin has numerous endocrinological effects (45,46), in addition to its well-characterized role in regulation of energy balance. Our study did not distinguish the obesity-promoting effects from its other endocrine effects. Therefore, we cannot formally attribute all of the diabetes related effects of leptin deficiency in our study to obesity.

Two types of interactions play a critical role in control of type 2 diabetes susceptibility in BTBR × B6 F2-*ob*/*ob* mice. First, a highly significant interaction between *ob* and strain reveals a large difference in diabetes susceptibility between BTBR-*ob*/*ob* and B6-*ob*/*ob* mice. The use of *ob* permitted the detection of two loci that are significantly linked to fasting plasma glucose or insulin traits. More importantly, another interaction between *t2dm1* and *t2dm2* greatly modifies risk of severe hyperglycemia in male F2 mice. This interaction helps explain why B6-*ob*/*ob* mice, which bear the diabetogenic *t2dm2*$^{B6}$ allele, are at relatively low risk for development of severe hyperglycemia. Our data suggest that the *t2dm1*$^{B6}$

allele prevents the *t2dm2*$^{B6}$ allele from increasing type 2 diabetes susceptibility.

In summary, we have used the *ob* allele to unmask latent differences in type 2 diabetes susceptibility between two mouse strains. In doing so, we uncovered surprising variation in the degree of β-cell compensation for the underlying insulin resistance. We discovered two major susceptibility loci and detected a nonlinear interaction between them that substantially increases risk of severe type 2 diabetes.

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