

# Identification of Interactive Loci Linked to Insulin and Leptin in Mice With Genetic Insulin Resistance

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Mice double heterozygous (DH) for deletion of insulin receptor and insulin receptor substrate-1 are lean, insulin resistant, and have a phenotype that strongly depends on the genetic background of the mouse. On the C57BL/6 (B6) background, DH mice develop marked hyperinsulinemia and diabetes, whereas on the 129S6 background, DH mice exhibit only mild elevations of insulin and remain free of diabetes. F2 male mice created by an intercross between these two strains exhibit a 60% incidence of diabetes and a bell-shaped distribution of insulin levels as related to glucose, reminiscent of that in humans with type 2 diabetes. These mice also exhibit a wide range of leptin levels as related to body weight. A genome-wide scan of F2 mice reveals a quantitative trait locus (QTL) related to hyperinsulinemia on chromosome 14 (*D14Mit55*) with a peak logarithm of odds (LOD) score of 5.6, accounting for up to 69% of this trait. A QTL with a LOD score of 3.7 related to hyperleptinemia is present on chromosome 7 at *D12Mit38* (a marker previously assigned to chromosome 12) in the area of the uncoupling protein 2/3 gene cluster. This locus also interacts synergistically with *D14Mit55* in development of hyperinsulinemia and with a QTL on chromosome 12 (*D12Mit231*) related to hyperglycemia. These data demonstrate how multiple genetic modifiers can interact and influence the development of diabetes and the phenotype of animals with genetically programmed insulin resistance and provide evidence as to the location and nature of these genes. *Diabetes* 52: 1535–1543, 2003

**T**ype 2 diabetes is a multifactorial disorder in which ~50–70% of cases can be attributed to genetic factors (1), with the rest being due to environmental factors (such as diet and lack of activity) that play major roles as modifiers of the preexisting genetic risk. Finding diabetogenes in the common forms of type 2 diabetes has been complicated because of the complexity and late onset of disease, the increased mortality of diabetic patients, and the fact that type 2

diabetes is often subclinical for many years and, consequently, the description of the family history of diabetes is only partially informative. Moreover, genetic research performed over the past few years suggests that type 2 diabetes is polygenic and may be caused by an epistatic interaction of a number of genes. Thus, examination of genotypes in very large populations would be essential to identify and define these genes and their interactions.

Although the common form of type 2 diabetes may be genetically heterogeneous, in most populations, insulin resistance is one of the earliest detectable defects (2). A genetic contribution to insulin resistance and type 2 diabetes has been demonstrated by a high heritability among first-degree relatives of diabetic patients, a higher concordance rate in monozygotic twins than in dizygotic twins, and clustering of diabetes in certain populations (3–5). Several genetic variants in insulin-signaling proteins have been identified as minor players in the development of insulin resistance and diabetes in some populations (6). With the exception of the relative rare forms of maturity-onset diabetes of the young (7), mitochondrial diabetes (8), and the type A syndrome of insulin resistance (9), the specific genes involved in most human diabetes remain unknown.

Analysis of diabetes genes in rodents offers several advantages over that possible in humans. These include the fact that a large number of animals can be bred for study, and thus both environmental influence and genetic heterogeneity can be diminished. In addition, the possibility of creating similar genetic lesions in mice of different background can be used to identify important modifiers of disease genes, and new mouse models of diabetes can be created by gene insertions or targeted gene disruptions. The creation of these latter models involves using mice of mixed genetic background; however, this may significantly affect the phenotype of the mice (10).

Some years ago, we created a polygenic animal model of type 2 diabetes by generating mice heterozygous for the insulin receptor (IR) and IR substrate-1 (IRS-1) null alleles (11). Whereas neither single allele created significant diabetes or insulin resistance, the double heterozygous (DH) mice exhibited an epistatic interaction with marked insulin resistance, such that 50% of the mice on a mixed genetic background of C57BL/6 (B6) and 129S6 developed diabetes by the age of 6 months. Recently, we backcrossed these genetically modified mice onto B6 and 129S6 backgrounds to obtain a genetic purity of ~98–99% (Kulkarni et al., accompanying article [11a]). Heterozygous modification of the IR and IRS-1 loci caused insulin resistance in both strains; however, the phenotypes were dramatically different. The backcrossed DH B6 mice became severely

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DH, double heterozygous; IR, insulin receptor; IRS-1, IR substrate-1; LOD, logarithm of odds; LRS, likelihood ratio statistic; PIAS, protein inhibitor of activated STAT (signal transducer and activator of transcription); QTL, quantitative trait locus; SNP, single nucleotide polymorphism; UCP, uncoupling protein.

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TABLE 1  
Impact of genetic background on glucose and insulin levels in wild-type and DH F1 outcross and F2 intercross mice

	B6 <sup>wt</sup>	129 <sup>wt</sup>	B6 <sup>DH</sup>	129 <sup>DH</sup>	F1 <sup>wt</sup>	F1 <sup>DH</sup>	F2 <sup>wt</sup>	F2 <sup>DH</sup>
<i>n</i>	101	101	102	101	11	16	71	79
Age (months)	6	6	6	6	2	2	6	6
Glucose (mg/dl)	146 ± 2	105 ± 2	297 ± 11	120 ± 3	134 ± 9	139 ± 8	137 ± 5	275 ± 16
Insulin (ng/ml)	17.1 ± 3.2	2.1 ± 0.3	259.5 ± 17.7	7.8 ± 1.0	ND	ND	ND	269.4 ± 29.9

Data are means ± SE. ND, not determined; wt, wild-type.

hyperinsulinemic with massive islet hyperplasia and developed early hyperglycemia and diabetes with an incidence of ~85% by 6 months. In contrast, the DH defect in 129S6 mice caused mild hyperinsulinemia and minimal islet hyperplasia, and <2% became diabetic. Thus, the inbred strain background can exert a powerful effect on the diabetogenic outcome. In the present study, we have taken advantage of these models to reveal the genetic modifiers responsible for the different phenotypes by creating and characterizing DH IR/IRS-1 F2 mice from an intercross between B6 and 129S6.

RESEARCH DESIGN AND METHODS

**Creation of F2 intercross mice.** Intercross mice were obtained by breeding IR/IRS-1 DH mice that had been previously backcrossed onto a B6 background for six to seven generations with 129S6 wild-type mice (strain 129S6/SvEvTac; Taconic, Germantown, NY) to create F1 outcross mice (Kulkarni et al., accompanying article [11a]). The F1 generation was then intercrossed in a random manner to obtain a total of 100 DH F2 mice, and female breeders were not used on more than two occasions. The breeding yielded approximately the expected numbers of genotypes. DNA was prepared from tail tips or kidney slices as previously described (11). The mice were maintained on a 12-h light-dark cycle and fed a diet containing 9% fat. All protocols for animal use were reviewed and approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines.

**Phenotype analysis.** Phenotype analyses were performed in 4- and 6-month-old male mice. Blood glucose values were determined from whole venous blood using an automatic glucose monitor (Glucometer Elite; Bayer, Mishawaka, IN). Diabetes was defined as random fed blood glucose levels >200 mg/dl. Insulin and leptin levels were measured in plasma samples by enzyme-linked immunosorbent assay using mouse insulin and mouse leptin as standards (Crystal Chem, Chicago).

**Genome-wide scan in F2 intercross mice.** Initially, 20 mice with the two extremes of the trait distribution (10 with the lowest glucose levels and 10 with the highest) were genotyped using 80 polymorphic markers covering the 19 autosomal chromosomes and the X chromosome (Research Genetics, Huntington, AL) with a proposed average distance of 20 cM. Genotypes were scored using 4% agarose gels or 10% polyacrylamide/urea gels. On the chromosomes where a marker showed a deviation from the expected Mendelian frequency, an additional 40 mice were genotyped (20 with low and 20 with high glucose levels) at 39 additional markers.

**Statistical analysis.** Data analysis was performed using Map Manager version QTXb15b (12), MapMaker/EXP 3.0, and MapMaker/QTL 1.1 (13–15). MapMaker/QTL calculates the strength of the association between markers and traits as the log base 10 of the odds ratio (logarithm of odds [LOD] score). The permutation test estimates an empirical genome-wide probability for a given likelihood ratio statistic (LRS). (Tests with 10,000 replications is the maximum number Map Manager performs. At least 100,000 replications may be required for this small dataset. The permutation test allows determination of critical significance values for interval mapping. The test gives three LRS scores: suggestive, significant, and highly significant, which correspond to the 37th, 95th, and 99.9th percentiles.) Statistical Package of Social Science (SPSS) for Windows (version 11.5) was used for the other statistical analyses and for the Jonckheere-Terpstra test to evaluate the interactive effects between loci. The Jonckheere-Terpstra test for differences among several independent samples is more powerful than the Kruskal-Wallis *H* or median tests (16,17). However, it requires that the independent samples be ordinally arranged on the criterion variable. The Jonckheere-Terpstra test tests the hypothesis that as one moves from samples low on the criterion to samples high on the criterion, the within-sample magnitude of the criterion variable

increases. A *P* value <0.05 (two-tailed) was considered significant. Both insulin and glucose levels in DH F2 intercross mice had an approximately normal distribution, whereas the leptin levels departed from a normal distribution.

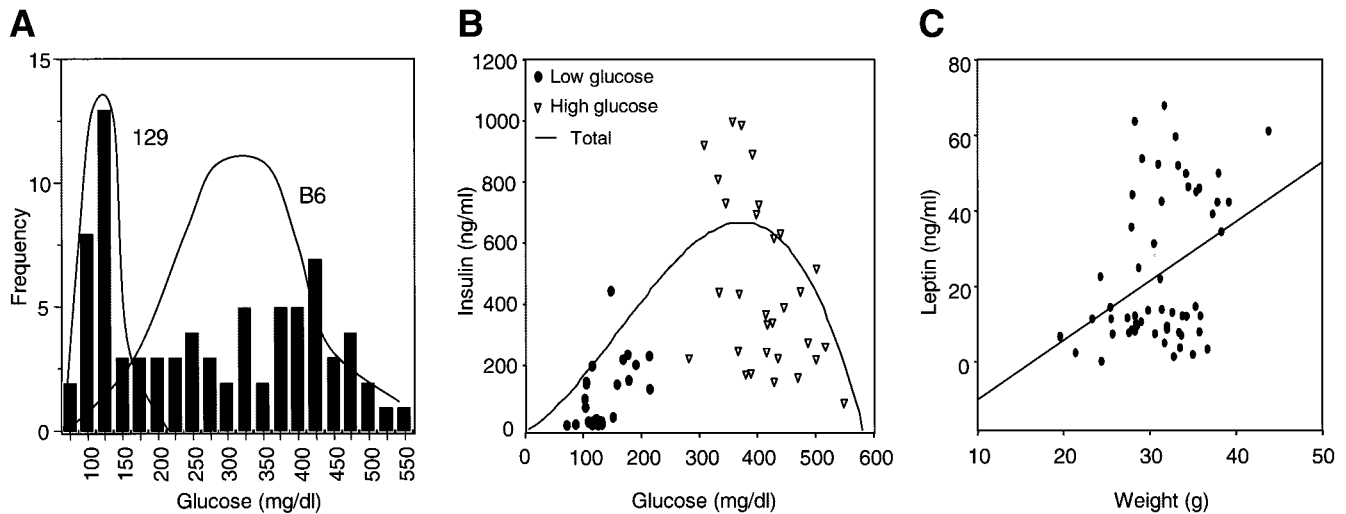
RESULTS

**Phenotypic characteristics of (B6 × 126S6) F2 intercross mice.** The IR/IRS-1 DH knockout mouse is a model of polygenic insulin resistance that is predisposed to development of diabetes in susceptible strains. To map genetic loci that may contribute to the different phenotypes observed in DH mice on the B6 and 129S6 genetic backgrounds, DH mice that had been backcrossed on the B6 background were mated with 129S6 wild-type mice to create an F1 outcross. Fed glucose levels in the F1 hybrids at 2 months averaged 139 mg/dl (Table 1) and were intermediate between the glucose levels in the two parental strains (B6 DH [glucose = 165 mg/dl] and 129S6 wild-type [111 mg/dl]), suggesting that the inheritance of this trait is not dominant. The F1 mice were intercrossed to obtain an F2 generation. In all generations and on all backgrounds, DH mice exhibited a reduction in size and body weight compared with wild-type mice, which on average was 21% because of the heterozygous IRS-1 deletion (18). For analysis of the F2 generation, only male mice were examined, because our previous studies have shown a greater degree of hyperinsulinemia and diabetes in the males (18).

Fed blood glucose levels in the DH F2 intercross mice ranged from 75 to >550 mg/dl with a mean of 275 mg/dl and with a bimodal distribution. The mean glucose level more closely resembles the mean glucose observed in the congenic DH B6 parental strain compared with that of the DH mice on the background of 129S6 (Table 1 and Fig. 1A). The variance of the F2 mice was substantially larger than the variance of the F1 mice (results not shown), indicating a segregation of genes affecting glucose levels. The incidence of diabetes (defined as fed blood glucose >200 mg/dl) at 6 months in the F2 intercross mice was 60%. Plasma insulin levels ranged from 4 to 1,000 ng/ml in DH F2 mice with a mean of 269.4 ng/ml. This was slightly higher than that observed in the B6 DH mice and considerably higher than the insulin levels in 129S6 DH mice (Table 1).

The relationship between fed insulin and glucose levels of the DH mice is shown in Fig. 1B. As has been observed in several studies of humans with type 2 diabetes (19) and some rodent models (20), this relationship fitted a quadratic model (*R*<sup>2</sup> = 0.50, *P* < 0.01), reflecting the fact that the insulin levels rise to a maximum at glucose levels of ~350 mg/dl but decrease toward normal above that glucose level. At any glucose, however, there was a wide

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**FIG. 1.** **A:** Histogram of blood glucose concentrations in IR/IRS-1 DH (B6  $\times$  129S6) F2 intercross mice at 6 months of age ( $n = 79$ ). The lines denoted 129 and B6 represent the normal curves of the glucose distributions of DH 129S6 and B6 backcross mice, respectively. **B:** Relationship between fed plasma insulin and blood glucose levels in IR/IRS-1 DH F2 intercross mice at 6 months of age. The scatterplot depicts the 60 mice ( $n = 30$  for each extreme of glucose levels) that were used in the genome scanning.  $\bullet$ , mice with low glucose levels;  $\nabla$ , mice with high glucose levels. **C:** Relationship between leptin and body weight in IR/IRS-1 DH F2 intercross mice at 6 months of age ( $n = 44$ ). The scatter plot depicts mice that were used in the genome scanning. The overall correlation of leptin to body weight was positive ( $R = 0.139$ ,  $P = 0.005$ ).

range of insulin values, suggesting a wide range of insulin resistance in the DH mice.

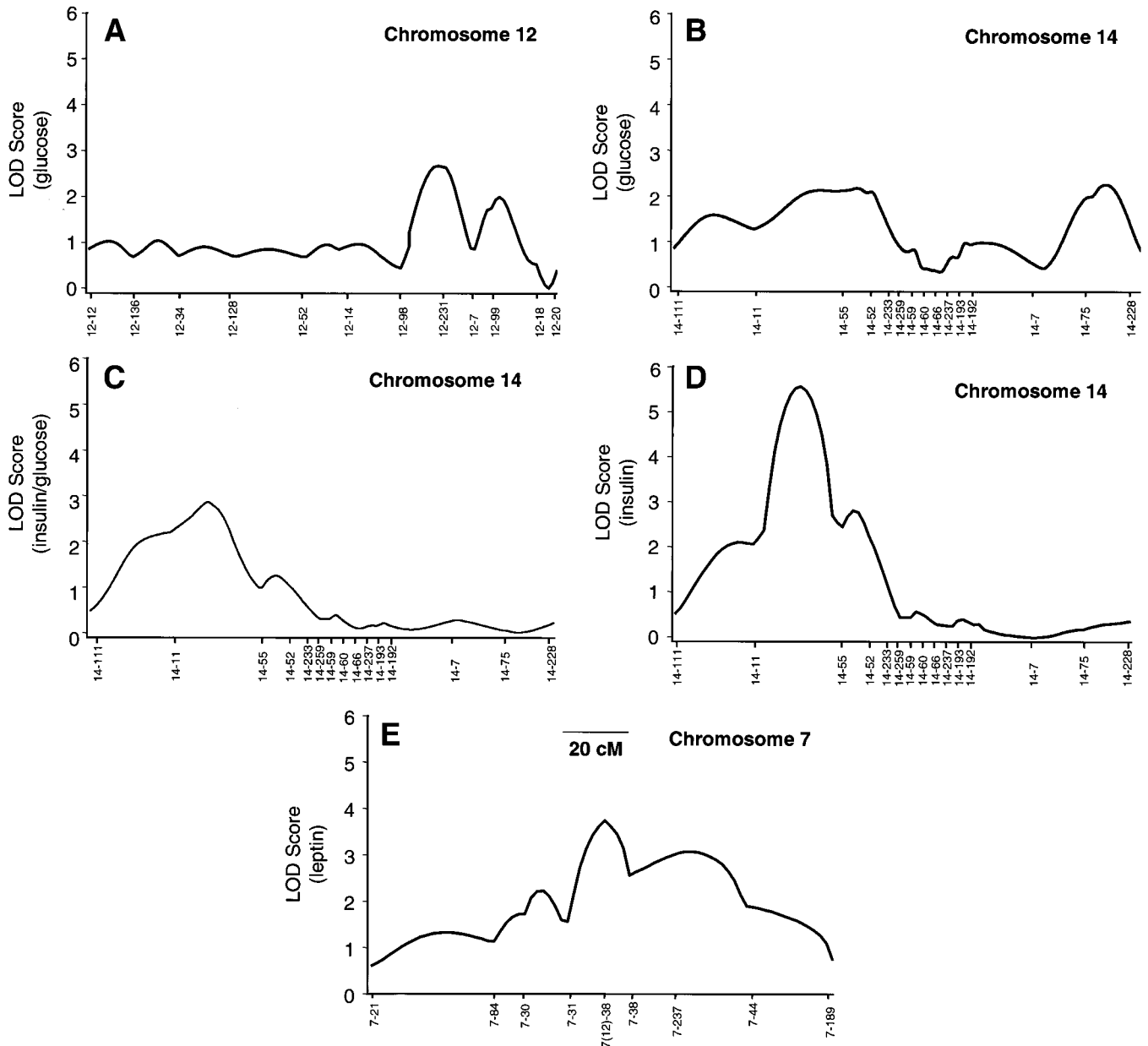
Leptin plays a critical role in appetite regulation and control of body weight homeostasis (21). Plasma leptin concentrations in both humans and rodents generally correlate to the body weight and total fat mass (21). In the DH F2 intercross mice, plasma leptin averaged 23.7 ng/ml ( $n = 57$ ) at 6 months. When plotted as a function of body weight, the plasma leptin levels exhibited an overall positive correlation to body weight ( $R^2 = 0.139$ ,  $P = 0.005$ ), although the range of values at any body weight was large, with some mice exhibiting very high leptin levels (Fig. 1C). The overall relationships between leptin and hyperglycemia, leptin and diabetes, and leptin and insulin were also all positive ( $P < 0.01$ ).

**Quantitative trait locus controlling glucose and insulin levels.** A linkage map of chromosome 12, based on genotyping data of 12 markers in 60 mice, was constructed by using both Map Manager (12) and MapMaker/EXP (13). The order of markers obtained independently from our data using both programs was similar to the Whitehead/Massachusetts Institute of Technology map, although the estimated distances between the markers on the map obtained from our analyses were slightly greater than those of the Whitehead/MIT map. We examined the linkage of glucose levels to regions on chromosome 12 by using MapMaker/EXP followed by MapMaker/QTL (Fig. 2A). MapMaker/QTL calculates the strength of the association between markers and traits as the log base 10 of the odds ratio (LOD score). The quantitative trait locus (QTL) evaluation of glucose levels at 6 months on chromosome 12 revealed a linkage peak with a LOD score of 2.7 at marker *D12Mit231*, explaining 26% of the phenotypic variance of the trait (Table 2 and Fig. 2A). Similarly, a linkage map of chromosome 14 based on genotyping data of 15 markers in 60 mice was constructed. The QTL analysis of glucose at 6 months revealed two peaks on chromosome 14 (Fig. 2B), a broad peak with a peak LOD score of 2.2 between marker *D14Mit52* and *D14Mit11*

(potentially representing more than one locus), and a narrower peak between *D14Mit75* and *D14Mit228* with a peak LOD score of 2.3 (Table 2). A QTL analysis of chromosome 12 and 14, using diabetes as a trait (defined as a fed blood glucose level  $>200$  mg/dl), revealed the same peak on chromosome 12 (*D12Mit231*) with a LOD score of 2.3 and a peak on chromosome 14 between marker *D14Mit11* and *D14Mit55* with a LOD score of 3.0 (Table 2).

Insulin resistance, as estimated by the ratio of insulin to glucose, demonstrated a QTL with a peak LOD score of 2.8 between *D14Mit11* and *D14Mit55* on chromosome 14 (Fig. 2C). To verify the QTL analyses, tests with 10,000 permutations were carried out using Map Manager. The permutation test estimates an empirical genome-wide probability for a given LRS (1). The permutation tests confirmed that the identified QTL with linkage to the insulin-to-glucose ratio and the QTL linked to glucose on chromosome 12 were significant (LRS score on chromosome 14 for the insulin-to-glucose ratio = 11.5 with significance at 11.2, and LRS score on chromosome 12 for glucose = 12.2 with significance at 12.0) (see RESEARCH DESIGN AND METHODS).

The strongest linkage in the QTL analyses was observed for insulin levels and occurred as a major peak on chromosome 14 between *D14Mit11* and *D14Mit55* with a LOD score of 5.6 (Fig. 2D). This region on chromosome 14 was estimated to account for up to 69% of the variance in insulin levels (Table 2). A permutation test also confirmed that this locus was significant (LRS score = 15.6 with significance at 11.2). The insulin levels of mice homozygous for the B6 allele at marker *D14Mit55* were significantly higher than the insulin levels of mice homozygous for the 129S6 allele ( $P = 0.018$ ), whereas the insulin levels of heterozygous mice were intermediate of the other two genotypes, suggesting a gene dosage effect (Fig. 3A). A smaller peak for insulin with a LOD score of 2.8 was located between *D14Mit55* and *D14Mit52*, similar to the location of one of the glucose peaks. QTL analysis of the



**FIG. 2.** *A:* QTL analysis of genotyping data on chromosome 12 revealed a locus linked to glucose levels in F2 mice at 6 months of age at marker *D12Mit231* with a peak LOD score of 2.7. *B:* Two loci with potential association to glucose were identified on chromosome 14 (a broad peak between marker *D14Mit55* and *D14Mit52* with a LOD score of 2.2 and a narrower peak between *D14Mit75* and *D14Mit228* with a LOD score of 2.3). *C:* QTL analysis of the insulin-to-glucose ratio revealed a peak with a LOD score of 2.8 between marker *D14Mit11* and *D14Mit55*. *D:* QTL analysis of insulin levels identified a peak with a LOD score of 5.6 between marker *D14Mit11* and *D14Mit55*. *E:* QTL analysis of chromosome 7 for leptin revealed a peak at marker *D7(12)Mit38* with a peak LOD score of 3.7. The genotyping was performed in 60 mice.

mean weight and leptin levels at 6 months did not show any evidence of linkage to the loci on chromosome 12 or 14.

**QTL controlling leptin levels.** DNA from 60 mice was also genotyped with nine markers on chromosome 7 to evaluate putative QTLs. The analyses of glucose, insulin, insulin-to-glucose ratio, and weight showed LOD scores <2. However, an analysis of leptin disclosed a QTL with a LOD score of 3.7 (Fig. 2E), with 33% of the phenotypic variance of the leptin trait explained at this locus (Table 2). The leptin peak was located around marker *D7(12)Mit38*, which was previously assigned to chromosome 12 in the MIT and Jackson Laboratory databases, but

is assigned to chromosome 7 in the Celera database (Celera, Rockville, MD). A linkage analysis of *D7(12)Mit38* with other markers on chromosome 7, using our own data, demonstrated a strong linkage, thereby verifying the location on chromosome 7. A permutation test confirmed that the identified QTL with linkage to leptin was highly significant (LRS score = 19.2 for leptin, with significance at 10.7, and highly significant at 18.7).

The leptin levels of mice homozygous for the B6 allele had significantly higher leptin levels than mice homozygous for the 129S6 allele ( $P = 0.027$ ). The leptin levels of heterozygous mice resembled the levels of the B6 homozygous mice, suggesting a dominant effect (B6 homozygous:

TABLE 2  
Results of QTL analysis of selected genotyping data

Chromosome	Marker	Trait	LOD score	Phenotypic variation (%)	Donor strain
12	D12Mit231	Glucose	2.7	26	B6
14	D14Mit55	Glucose	2.2	21	B6
14	D14Mit75	Glucose	2.3	23	B6
12	D12Mit231	Diabetes	2.3	24	B6
14	D14Mit55	Diabetes	3.0	31	B6
14	D14Mit55	Insulin/glucose	2.8	46	B6
14	D14Mit55	Insulin	5.6	69	B6
7	D7(12)Mit38*	Leptin	3.7	33	B6

Genotyping data were analyzed using MapMaker/QTL. The indicated markers denote the marker closets to the peak. Phenotypic variation indicates the percentage of the phenotypic variation of the trait that can be explained by the locus. \*Initially assigned to chromosome 12.

leptin = 32.5 ng/ml; heterozygous: leptin = 30.5 ng/ml; 129S6 homozygous: leptin = 14.2 ng/ml). Moreover, when expressed as a function of body weight, mice homozygous for the 129S6 allele exhibited only minor increases in leptin levels with increasing body weight, whereas mice homozygous for the B6 allele exhibited a much larger increase in leptin with increasing body weight (Fig. 3B).

**Sequencing of uncoupling protein 2 and 3 encoding genes.** The region of the leptin QTL on chromosome 7 overlaps the genes for uncoupling protein (UCP)2 and UCP3. To determine whether any genetic variation were present in UCP2 and UCP3, we sequenced the six coding exons of both genes as well as large parts of the introns and 5'-untranslated regions on DNA from two B6 and two 129S6 mice. Two single nucleotide polymorphisms (SNPs) were identified in the intronic region of UCP2 (at nucleotide 4554 and 5158), and one SNP was identified in an intron (nucleotide 15442) of UCP3. The relationship between leptin and weight according to the SNPs showed a similar pattern as the data presented in Fig. 3B.

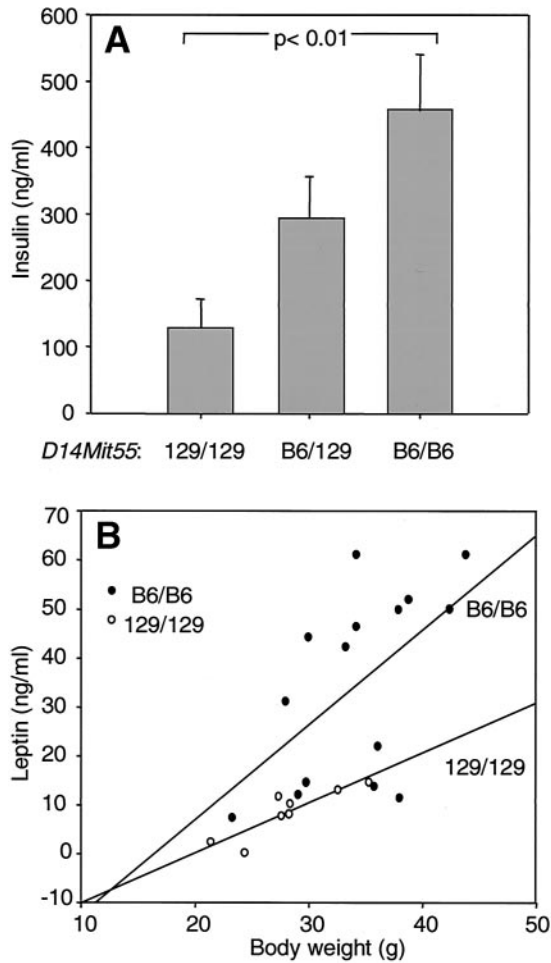
**Interactive effects of identified loci.** A potential interactive effect of the identified QTLs in the genetic control of susceptibility to hyperglycemia, hyperinsulinemia, and hyperleptinemia were investigated by pairwise analyses. With respect to the level of hyperglycemia, there was an interaction between the hyperglycemia locus on chromosome 12 (*D12Mit231*) and the locus on chromosome 7 [*D7(12)Mit38*] linked to leptin. Thus, even for mice homozygous for the B6 allele at *D12Mit231*, there appeared to be a further gene dose-dependent increase in glucose levels associated with the presence of the B6 allele at *D7(12)Mit38* (Fig. 4A), although this trend was only of borderline statistical significance ( $P = 0.055$ ) by the Jonckheere-Terpstra test, a nonparametric test for ordered differences among classes (see RESEARCH DESIGN AND METHODS) (16,17). Moreover, whereas homozygosity for B6 at marker *D14Mit55* was associated with hyperinsulinemia (Fig. 3A), this effect was further enhanced by B6 homozygosity at the loci identified on chromosome 7 [*D7(12)Mit38*] (Fig. 4B). The interaction between these two loci on the insulin levels was highly significant ( $P = 0.008$ , Jonckheere-Terpstra test). Finally, homozygosity for B6 at marker *D7(12)Mit38* and the potential hyperglycemia locus on chromosome 12 (*D12Mit231*) also exerted a synergistic effect on the leptin levels accentuated by the

additional B6 alleles, which also reached statistical significance ( $P = 0.016$ ) (Fig. 4C).

## DISCUSSION

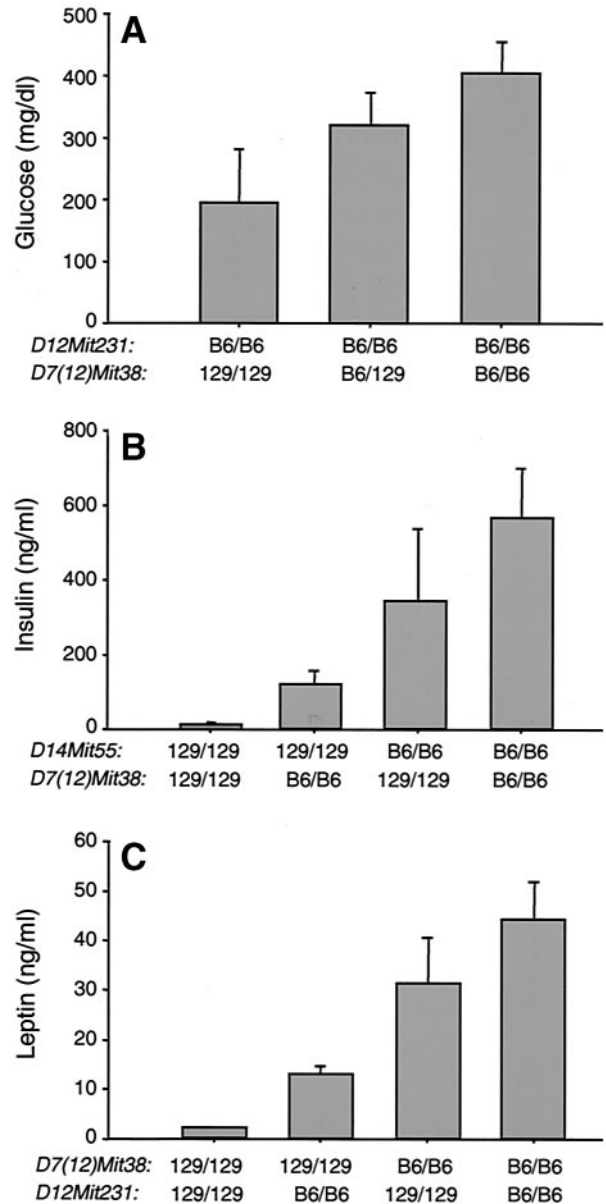
Type 2 diabetes in humans is a complex disease that is both polygenic and heterogenous with respect to etiology (6). The IR/IRS-1 DH knockout mouse provides some similarities to the human situation. Our recent studies have suggested that there is an epistatic interaction between the IR/IRS-1 heterozygous state and background genes from the B6 strain that causes a worsening of the genetically induced insulin resistance and, consequently, the development of diabetes, whereas the IR/IRS-1 heterozygosity on a 129S6 background causes minimal hyperinsulinemia and rarely causes diabetes (Kulkarni et al., accompanying article [11a]). In an effort to understand the role of genetic variation, we have used F2 intercross mice (B6  $\times$  129S6) of this polygenic model of insulin resistance as a tool to unmask loci that contain modifying diabetogenes. The F2 DH mice exhibit a very wide range of glucose and insulin levels, consistent with a nondominant mode of inheritance, similar to human diabetes. Furthermore, when insulin is plotted against glucose levels for the IR/IRS-1 DH mouse model, there is an inverted U-shaped relationship reminiscent of the progression of human impaired glucose tolerance to type 2 diabetes (22). Thus, the IR/IRS-1 DH knockout mouse is similar to human type 2 diabetes with a polygenic etiology, genetically programmed insulin resistance, a delayed age of onset, and a biphasic relationship between insulin and glucose levels, which is strongly modified by background genes. This provided us with an opportunity to begin to define these genetic modifiers.

When the DH F2 intercross mice were divided into two groups based on low and high glucose levels, a genome-wide scan revealed an excess of B6-derived alleles on chromosome 14 in mice with high glucose levels. QTL analysis identified two potential loci on this chromosome (peak LOD scores  $<3$ ) linked to hyperglycemia. The genetic analysis also revealed a QTL linked to hyperinsulinemia on chromosome 14 with a peak LOD score of 5.6 accounting for up to 69% of the variation of this trait. Thus, the genetic effect around marker *D14Mit55* seems to explain a large part of the difference between the strains.



**FIG. 3. A:** Insulin levels in mice according to genotype at marker *D14Mit55* on chromosome 14. The insulin levels of mice homozygous for the B6 allele were significantly higher than insulin levels of mice homozygous for the 129S6 allele. **B:** Relationship between leptin and weight according to genotype of marker *D7(12)Mit38* on chromosome 7. ●, Mice homozygous for the B6 allele; ○, mice homozygous for the 129S6 allele. All mice with homozygosity for the 129S6 allele exhibited minor increases in leptin levels with increasing body weight, whereas mice homozygous for the B6 allele on average exhibited a much larger increase in leptin with increasing body weight. The leptin levels of B6 homozygous mice were significantly higher than leptin levels of 129S6 homozygous mice ( $P = 0.027$ ). The heterozygous mice resembled the leptin levels of the B6 homozygous mice (129S6 homozygous: leptin =  $14.2 \pm 5.9$  ng/ml; heterozygous: leptin =  $30.5 \pm 4.6$  ng/ml; B6 homozygous: leptin =  $32.5 \pm 4.8$  ng/ml). A single F2 mouse exhibited a very high leptin concentration (59.6 ng/ml) and carried the 129S6 genotype in the identified region. This mouse was an exception to the rule, suggesting that he had additional recombination or interaction events leading to more complex genetics and was therefore excluded.

None of the loci identified on chromosome 14 overlaps with any known insulin resistance or type 2 diabetes genes in mice or rats, and to our knowledge, no previous reports have been made of QTLs in mice with linkage to insulin or glucose on chromosome 14. However, there are several interesting candidate genes in the identified region in public and the Celera database that could be linked to insulin resistance. Thus, a gene for PIAS (protein inhibitor of activated STAT [signal transducer and activator of transcription]), overlaps the locus linked to hyperinsulinemia. STAT proteins become activated by tyrosine phosphorylation in response to cytokine and insulin stimulation (23), and the activated STAT can be inhibited by PIAS (24).



**FIG. 4.** Interactive effect of the identified QTLs in the genetic control of susceptibility to hyperglycemia (A), hyperinsulinemia (B), and hyperleptinemia (C). The effect on glucose levels due to the interaction between marker *D12MIT231* linked to glycemia and *D7(12)Mit38* linked to leptin are shown in A ( $P = 0.055$ ). The effect on insulin levels due to the interaction between marker *D14MIT55* linked to insulin levels and *D7(12)Mit38* linked to leptin are shown in B ( $P = 0.008$ ). The effect on leptin levels due to the interaction between marker *D7(12)Mit38* linked to leptin and *D12MIT231* linked to glycemia are shown in C ( $P = 0.016$ ).

The region also overlaps protein kinase C- $\delta$ , one of several serine-threonine protein kinases that has been implicated in action, activity, and intracellular trafficking of the IR (25). Other genes of potential interest in the region are the genes for Arf4 (ADP-ribosylation factor 4), which has been implicated in GLUT4 trafficking (26) and Dusp13 (dual-specificity phosphatase 13) belonging to the family of phosphatases that can act on both serine and tyrosine phosphorylated proteins (27).

It is also interesting to note that the loci detected in the study are different from those identified in the genome-

wide scan of F2 intercross (B6 × 129S6) mice with heterozygosity for the IR null allele only (28). These IR (+/−) mice exhibit only very mild hyperinsulinemia, and most have glucose levels similar to the B6 wild-type mice, although ~15% of the F2 IR heterozygous mice develop diabetes. Scanning of these mice revealed QTLs on chromosome 2 (*D2Mit151*) and 10 (*D10Mit42*; now reassigned to chromosome 11), with significant linkage to insulin and with B6 as the donor strain. Two other markers on chromosome 1 (*D1Mit19*) and 12 (*D12Mit231*) showed suggestive linkage to insulin. The alleles were contributed from the B6 and 129S6 strains, respectively (28). In our study, *D12Mit231* is potentially linked to hyperglycemia, with B6 as the donor strain. No evidence of linkage to either insulin or glucose for the other potential QTLs identified in the IR heterozygous F2 mice was observed in our DH mice. The differences between these two studies suggest that addition of IRS-1 heterozygosity to IR heterozygosity not only creates a synergistic (epistatic) effect in the development of diabetes, but also unmasks additional interacting genetic modifiers that are not observed in the IR heterozygous-only knockout mouse.

Other studies have been performed in an effort to determine the contribution of genetic background in mice. An intercross between CAST and B6 mice showed obesity loci on chromosome 2 and linkage to insulin levels with alleles from the B6 strain (29). When the *ob* gene was introduced into BTBR mice to create an F2 intercross between BTBR-*ob/ob* and B6-*ob/ob* mice, a locus on chromosome 19 was identified with significant linkage to low fasting insulin and suggestive linkage to high glucose with the alleles derived from the B6 strain (30). The locus exhibited a nonlinear interaction with a locus on chromosome 16 derived from the BTBR strain, which together caused a substantial increased risk of type 2 diabetes. None of these QTLs were observed in our model.

Leptin plays a role in controlling appetite and energy metabolism (21). Because the B6 strain is prone to obesity (31–34) (K.A., C.R.K., unpublished data), we were intrigued by the identification of a QTL with linkage to leptin contributed from the B6 strain. Thus, when the DH F2 mice are divided into two groups based on the genotype at *D7(12)Mit38*, mice carrying B6 alleles exhibit a marked increase in leptin levels with increasing body weight, whereas mice carrying 129S6 alleles exhibit a more modest increase of leptin with increasing weight. Whether this represents differences in leptin sensitivity or a different relationship between leptin and body weight is unknown.

The chromosome 7 locus affecting leptin overlaps the genes for UCP2 and UCP3 and is orthologous to the locus on human chromosome 11q13, which contains the UCP2 and UCP3 gene cluster. The UCPs are mitochondrial proteins acting as uncouplers of oxidative phosphorylation and thereby converting fuel into heat instead of ATP (35). A connection between leptin and UCP2 and UCP3 has been previously observed in several studies. Continuous leptin administration in rats increases the mRNA expression of UCP2 and UCP3 and prevents the decrease in their expression that occurs during fasting (36–38). Acute leptin treatment in B6 mice, on the other hand, is associated with a decrease in UCP2 and UCP3 mRNA expression in white adipose tissue and skeletal muscle,

respectively (39). A difference in diet-induced expression of UCP mRNA has also been demonstrated between obesity-resistant A/J mice and obesity-prone B6 mice (31,34,40). Similarly, a high-fat diet has been shown to result in a fivefold higher expression of UCP2 mRNA in white fat of 129S6 mice compared with B6 mice (E. Kokkotou, E. Maratos-Flier, personal communication). There is also suggestive evidence for a locus on chromosome 7 containing the UCP2 and UCP3 gene cluster affecting adiposity in F2 mice from an intercross between CAST/Ei and B6 mice fed a high-fat diet (41) and QTLs with linkage to adiposity, total cholesterol, and carcass lipid in a similar region (42,43).

Although genetic variation in UCP2 or UCP3 are not associated with any major alterations in body weight in humans (44), a linkage between markers in the vicinity of the UCP2 gene and the resting metabolic rate is suggested in a study of pedigrees from the Quebec Family Study (45). A recent study has also reported that UCP3 protein content in humans with type 2 diabetes is ~50% the level found in healthy control subjects (46).

The exact relationship between leptin, UCP2 and UCP3, and regulation of body weight needs further examination; however, based on our current knowledge, we propose that a “resistance” to leptin-induced expression of either UCP2 or UCP3 caused by genetic alterations in the genes or their promoter in the B6 mouse strain could result in feedback and cause an increase in leptin concentrations. If the regulation of UCP2 and UCP3 by leptin is defective, this may cause a decrease in the expression of the proteins and, consequently, a decrease in energy expenditure, which in the long run would result in slightly increased fat and a higher incidence of diabetes, especially in the context of IR/IRS-1 heterozygosity.

Based on our results and these previous studies, we sequenced the UCP2 and UCP3 genes. Only intronic polymorphisms were identified; however, the relationship between leptin and body weight according to the UCP2 and UCP3 SNP genotype showed a similar pattern as *D7(12)Mit38*, confirming that the UCP2 and UCP3 gene cluster is located in the identified locus. A more extensive analysis of upstream untranslated sequences will be necessary to determine whether allelic variants of either of these genes might be responsible for the differences of the leptin levels among the DH mice and the development of diabetes.

As with all complex genetic diseases, interaction between loci could be critical in the genetic determinants of the phenotype. In the DH mice, the QTL on chromosome 14 (*D14Mit55*) show a significant increase in insulin with increasing B6 allele number and an interaction with the QTL at *D7(12)Mit38*. Thus, alleles from the 129S6 mice at *D7(12)Mit38* suppress hyperinsulinemia, whereas the B6 alleles interact with the QTL for insulin in an additive manner and causes an increase of the insulin levels. Likewise, the potential QTL linked to hyperglycemia at *D12Mit231*, contributed by the B6 strain, also interacts with the QTL at *D7(12)Mit38*, such that addition of two B6 alleles at marker *D7(12)Mit38* causes the glucose levels to double. The same two QTLs also interact and affect the leptin levels. Thus, the effect of the QTL at *D12Mit231* becomes significant as a result of the interac-

tion with other loci. Although, we have to be cautious in the interpretation of these results because of the moderate number of mice in each group. A combined action of not only susceptibility but also suppressive QTLs appear to determine the outcome of the traits seen in the DH mice, further reflecting the nature of this polygenic disease.

In conclusion, we have identified a novel QTL linked to hyperinsulinemia on chromosome 14 and a locus on chromosome 7 in the region of UCP2 and UCP3 that is linked to hyperleptinemia. These loci also interact in the development of hyperglycemia, hyperinsulinemia, and hyperleptinemia. Further genetic mapping of mouse and human chromosomes may disclose the potential diabetogenes in the regions and may be helpful in the continuing search for candidate genes in human type 2 diabetes.

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The proposed order of the microsatellite markers on chromosome 14 has recently been revised in the Celera Database. According to the current order of markers the LOD score for hyperinsulinemia is ~3 and still significant. Furthermore, the insulin locus on chromosome 14 has been replicated in another study of insulin resistance in B6x129 intercross mice (K.A., C.R.K., unpublished data).

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