

Mapping of Murine Diabetogenic Gene *Mody* on Chromosome 7 at D7Mit258 and Its Involvement in Pancreatic Islet and β Cell Development during the Perinatal Period

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Abstract

Mutation of the murine maturity-onset diabetes mellitus of the young (*Mody*) locus induces diabetes, but the effects of its homozygosity on the pancreas remain unknown. F2 mice were obtained by F1 (diabetic C57BL/6 \times normal *Mus musculus castaneus*) crosses. About 20% of the F2 progeny developed diabetes by 2 wk of age, 50% of the progeny were normal at 2 wk and developed diabetes between 5 and 8 wk of age, and the remaining 30% did not develop diabetes. Quantitative trait locus analysis using blood glucose levels of 118 F2 mice at 2 wk of age and 5–8 wk of age located *Mody* within 3 cM of D7Mit258. Histopathological investigation revealed hypoplastic islets (\sim 33% of that of wild-type mice) and a lower density of β cells (\sim 20% of wild-type) with a reciprocal dominance of α cells (four times that of wild-type) in *Mody* homozygotes. Electron microscopic observations revealed a specific decrease in the number of insulin secretory granules and a lower density of β cells. Ratios of insulin to glucagon contents confirmed specific decreases in insulin content: 0.01 for homozygotes, 0.54 for heterozygotes, and 1.11 for wild-type mice on day 14. These results suggest that *Mody* is involved in both islet growth and β cell function. (*J. Clin. Invest.* 1998. 101:2112–2118.)
Key words: homozygosity • neonatal diabetes • *Mody* • quantitative analysis • inborn

Introduction

Diabetes mellitus, both insulin-dependent (IDDM)¹ and non-insulin-dependent, is one of the great health concerns of modern societies (1). The prevalence of diabetes is estimated to be

5–10% of the total population worldwide (1). Prevention, clinical management, and countermeasures against the progression of diabetes have become a major health care concern. However, the genetics and physiology of diabetes are still poorly understood because of the multifaceted nature of human diabetes (2, 3).

We established recently the Akita mouse, a mutant line (derived from C57BL/6N) which spontaneously develops early age-onset diabetes mellitus without insulinitis or obesity. This trait is characterized by an autosomal dominant mode of inheritance (4). The clinical features of the Akita mouse diabetes are very similar to those of human maturity-onset diabetes mellitus of the young (MODY) (5). In affected mice, specific decreases in β cell density in the islets and hypoinsulinemia were observed (4). The responsible genetic locus of this disease, *Mody* (we have changed the mouse locus name, originally *Mody4*, to *Mody* to avoid confusion with the newly established diabetic locus in humans named MODY4 [6]), has been found previously to be located in the telomeric region of chromosome 7 (4).

The aim of this study was twofold. First, we sought to determine the dosage effect of the *Mody* mutant gene in the homozygous state. The *Mody* locus has been maintained in the heterozygous state (*Mody* +/-), and it therefore remains unknown whether homozygosity (*Mody* +/+) is lethal or associated with developmental anomalies. In addition, the dosage effects of the homozygous condition should delineate the cytological target as well as the physiological consequences. The second aim was to map *Mody* with high resolution. F1 (C57BL/6 \times *Mus musculus castaneus*) crosses result in greater polymorphisms in the genetic makeup and thus will enable us to map the locus with high resolution by the analysis of quantitative trait locus (QTL).

We report herein that the *Mody* locus was proven to have tight linkage with the D7Mit258 marker on chromosome 7. Homozygous mice (*Mody* +/+) were found to develop diabetes by 2 wk of age and to have inborn hypoplasia of islets accompanied by β cell hypoplasia. These results suggest that the cytological target of the mutated *Mody* gene product is β cells, thereby affecting islet growth and development in the neonatal period.

Methods

Akita mice and animal care. The diabetic mice, C57BL/6 (B6) background, have been bred in our vivarium since 1993 (4). Stock colonies were maintained by mating diabetic males to nondiabetic outbred C57BL/6N Jcl females from the Nippon Clea colony. Mice were weaned at 3 wk of age and housed in cages with wood shavings in a room with a relative humidity of 50% and a 12-h light (0700–1900)/12-h dark (1900–0700) photocycle. The ambient air temperature in the animal rooms was kept at 20–22°C. All mice were handled in accordance with the Animal Welfare Guidelines of Akita University (1990). Mice were given free access to food (commercial lab chow,

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Received for publication 26 September 1997 and accepted in revised form 12 March 1998.

1. Abbreviations used in this paper: B6, C57BL/6; IDDM, insulin-dependent diabetes mellitus; ipGTT, intraperitoneal glucose tolerance test; LOD, logarithm of the likelihood; MODY, maturity-onset diabetes mellitus of the young; QTL, quantitative trait locus.

J. Clin. Invest.

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0021-9738/98/05/2112/07 \$2.00

Volume 101, Number 10, May 1998, 2112–2118
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CE-2; Nippon Clea, Tokyo, Japan) and water. The CE-2 diet contains (wt/wt%) 4.4% fat, 24.8% protein, 51.6% carbohydrate, and a total energy content of 1,445 J/100 g of diet.

F2 mice and phenotyping. Diabetic female B6 mice from the stock colony (referred to as F0) were mated to castaneus/Ei (*M.m. castaneus*) from either The Jackson Laboratory (Bar Harbor, ME) or the National Genetic Research Center of Japan (Dr. T. Shiroyshi, Mishima, Japan). The male castaneus/Ei had normal morning blood glucose levels and were not diabetic. F1 mice were crossed to obtain F2 mice.

Diabetic phenotypes were determined by morning blood glucose levels or by the results of the intraperitoneal glucose tolerance test (ipGTT). The ipGTT was performed by injecting glucose (1.5 g/kg in 15% solution) i.p. in mice after overnight fasting (~16 h) (4). Blood glucose levels were determined before glucose injection and at 30, 60, and 120 min after injection.

Diabetic male mice were defined as having morning blood glucose levels > 16.7 mmol/liter. For female mice, the criteria included (a) morning blood glucose levels > 11.1 mmol/liter, or (b) impaired ipGTT, > 20.0 mmol/liter at 30 min, 16.7 mmol/liter at 60 min, or 11.1 mmol/liter at 120 min. Blood glucose levels were determined using a Tidx[®] monitor (Bayer Corp., Tarrytown, NY). These reference values for each gender were the corresponding means of unaffected B6 mice \pm 2SD. Some of the female mice were phenotyped by ipGTT between 7 and 8 wk of age. The F2 mice were divided into three groups, the cohort group, a group for pathological investigation, and a group for determining gene frequency. Mice of the first group were observed until 8 wk of age for quantitative trait locus (QTL) analysis. The mice of the second group were killed for pathological investigation within 24 h after delivery or at 2 wk of age. The last group was killed immediately after delivery.

Genotyping. DNA samples were prepared from the blood or livers of F2 progeny using a DNA extraction kit (Ready Amp genomic DNA purification system; Promega Corp., Madison, WI). Microsatellite markers were typed by amplifying regions (100–300 bp) containing simple sequence length variants using PCR. Primer pairs were synthesized for microsatellites that were polymorphic between B6 and castaneus. Mice were genotyped with seven microsatellite markers in the telomeric region of chromosome 7, where the *Mody* locus was assigned previously (4). The markers were D7Mit292 (69 cM), D7Mit242 (69 cM), D7Mit362 (72.4 cM), D7Mit177 (72.4 cM), D7Mit258 (72.4 cM), D7Mit259 (72.4 cM), and Nds4 (72.4 cM). The microsatellite sequences and locations were obtained from Mouse Genome Informatics (The Jackson Laboratory, <http://www.informatics.jax.org>).

The PCR amplification reaction mixture contained 50 mM Tris-HCl, pH 9.5, 20 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 40 μ M of each deoxynucleotide triphosphate, 1 mM of each primer, and 50 ng of DNA in a final reaction volume of 15 μ l. PCR was performed using a programmable thermal controller (model PTC 100; MJ Research, Watertown, MA). The reaction was “hot started” at 95°C for 9 min after which 1.5 U of *Taq* Gold polymerase (Perkin-Elmer Corp., Branchburg, NJ) was added. The samples were amplified using a program of 45 s at 94°C, 45 s at 50°C, and 1 min at 74°C for 40 cycles. After the last cycle, the temperature was held at 72°C for 7 min. Microsatellite marker polymorphisms were analyzed by electrophoresis on 2% agarose gels (MetaPhor agarose; FMC Corp., Rockland, ME). Data were stored and organized using Map QTL Manager (version 2.11), kindly provided by Dr. K. Manly (Roswell Park Center Cancer Institute, Buffalo, NY). A complete linkage map was constructed for F2 crosses using an algorithm installed in Map QTL Manager. The allelic polymorphism of B6 was designated as the B allele, and that of Castaneus as the C allele.

Linkage and QTL analysis. Determinations of blood glucose levels were started at 2 wk of age. QTL analysis was conducted using the blood glucose levels at 2 wk of age and the mean blood glucose values between 5 and 8 wk of age. Genotype data and blood glucose values were analyzed using the QTL mapping method as implemented in the

Map QTL Manager. Mice that died before 2 wk were excluded from analysis. The blood glucose values obtained at the oldest age were used in place of the mean value for those mice that died between 2 and 5 wk of age. If possible, the means of available data were used for mice that died between 5 and 8 wk. Results of QTL analysis were expressed as logarithm of the likelihood (LOD) scores. To define the LOD score for this experiment, type I error of 0.01, a new set of 1,000 permutation traits was derived from the blood glucose data by random shuffling. The LOD score of each permuted trait analysis was determined, and the 99th percentile of the whole set was defined as the LOD score threshold.

Pathological investigations of islets. Mice were killed with pentobarbital (50 mg/kg) within 24 h of birth (age 1 d) or 2 wk after delivery (2 wk of age) for pathological investigations. Blood glucose levels were determined before killing. DNA samples were collected from the liver for genotyping D7Mit258. The entire pancreas was removed and divided into two pieces. One piece was fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, at 4°C. After determination of genotype, four pancreatic tissues per gender and per age group were selected randomly, a total of 48 samples, and processed further for electron microscopic observations. The other pieces of corresponding samples were fixed for immunohistochemistry in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 d at 4°C. After cryoprotecting in 20% sucrose and embedding in OCT compound (Miles Inc., Elkhart, IN), the specimens were frozen rapidly with liquid nitrogen. Sections (6 μ m) were cut using a cryostat (OT/FAS-002; Bright Instrument Co., Ltd., Huntingdon, UK) and placed on gelatin-coated slides. Slices for immunochemical analysis were treated with 50 mM NH₄Cl in PBS for 20 min and then immunostained.

To identify α and β cells, the fixed sections were first immunostained with the primary antibodies guinea pig antiinsulin (DAKO Japan Co., Ltd., Kyoto, Japan) and rabbit antiglucagon antisera (Linco Research, Inc., St. Charles, MO) followed by staining with FITC-conjugated donkey anti-guinea pig IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and with indocarbocyanide-conjugated affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.), respectively. The specimens were examined using a laser scan microscope (LSM410; Carl Zeiss, Oberkochen, Germany). All islets in four different whole sections per animal stained with hematoxylin and eosin were measured (~20 islets per mouse). Islet areas were measured by computerized image analyzing techniques using NIH Image software on a Macintosh, kindly provided by Dr. W. Rasband (National Institutes of Health, Bethesda, MD). The areas of all islets in each section were added together and divided by the total area of the section for determination of islets/(exocrine + islets) in the tissue. Immunological insulin- and glucagon-reactive areas were determined in each islet in four different whole sections per animal (~20 islets per mouse). The proportion of insulin- or glucagon-reactive areas was obtained by dividing by the total area of islets.

Insulin and glucagon contents in the pancreas. Mice were killed with pentobarbital (50 mg/kg) within 24 h of birth (age 1 d) or 2 wk after delivery (2 wk of age) for determination of insulin and glucagon contents in the whole pancreas. Pancreases were dissected and kept in an aqueous acid-ethanol solution (75% ethanol/12 mM HCl) overnight at 4°C as described by others (7). The extracts were then centrifuged, and the supernatants were kept at -20°C until assayed for insulin and glucagon. Insulin and glucagon concentrations were determined by RIA with insulin kits (Eiken Chemical Co. Ltd., Tokyo, Japan) and glucagon kits (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan). DNA samples were collected from the liver for genotyping D7Mit258.

Statistical analysis. Values were expressed as means \pm SD or \pm SEM. Statistical analysis was performed by ANOVA using a StatView Software package run on a Macintosh. Insulin and glucagon contents per pancreas were found to distribute normally on a log scale, and thus, statistical analysis was carried out on the transformed data.

Table I. Results of Linkage Analysis of the *Mody* Locus in the F2 Generation with Seven Microsatellite Markers Located on the Telomeric Region of Chromosome 7

Markers	Allele*		Crosses	n [‡]	Map	SEM	LOD
	C	B					
D7Mit292	141	87					
D7Mit242	139	85	3	112	1.34	0.77	60.5
D7Mit258	138	98	3	112	1.34	0.77	60.5
<i>Mody</i>	138	98	0	118	0.00	0.00	71.0
D7Mit362	140	90	6	115	2.63	1.05	57.2
D7Mit177	139	91	1	115	0.44	0.43	66.4
D7Mit177	139	91	3	114	1.32	0.75	61.7
D7Mit259	136	92	1	114	0.44	0.44	65.8
Nds4	137	97					

*C, the allele for Castaneus; B, the allele for B6. [‡]Number of informative F2 mice. The order of loci was obtained to minimize crosses using Map QTL Manager as described in Methods.

Results

Mapping *Mody*. 17 deliveries resulted in the birth of 135 F2 neonates, 7.94 ± 1.95 neonates per litter. These F2 neonates were observed until 8 wk of age. 17 (12.6%) of the neonatal mice died before 2 wk of age, and the genotypes of these mice were not determined. 57 males and 61 females survived to 2 wk of age, at which time monitoring of blood glucose levels was begun. Blood glucose levels were monitored once a week thereafter. We observed 11 deaths between 2 and 8 wk, nine (four males and five females) of which had hyperglycemia (28.1 ± 5.1 mM, 19.4–33.3 mM); the remaining two neonates had normal blood glucose levels (9.2 and 10 mM). The cause of death for the nine mice in the first category was considered to be due to hyperglycemia, but the cause of death for the others was unknown. The mortality rate of mice with hyperglycemia (9 of 22 mice) was significantly higher than for those without hyperglycemia ($\chi^2 = 32.6$, $P < 0.0001$). Genotyping at D7Mit258 revealed that the nine hyperglycemic mice had BB genotype and the other two mice had CC genotype.

To map *Mody*, we first constructed a linkage map using seven polymorphic microsatellite markers that cover the putative *Mody* locus. The order and map distances of the seven loci were determined (Table I). The whole map distances among markers were 7.3 cM. These markers were tightly linked, with high LOD scores (> 57.2).

As shown in Fig. 1, three phenotypes could be distinguished in the F2 generation: 22 of 118 mice developed diabetes at 2 wk of age (the early diabetic phenotype), 55 of the remaining 96 mice developed diabetes between 5 and 8 wk of age, and the remaining 41 mice did not develop diabetes. It was also of note that body weight gain was depressed in the early diabetes phenotype mice. We used two quantitative traits for QTL analyses. As shown in Fig. 2, blood glucose levels at

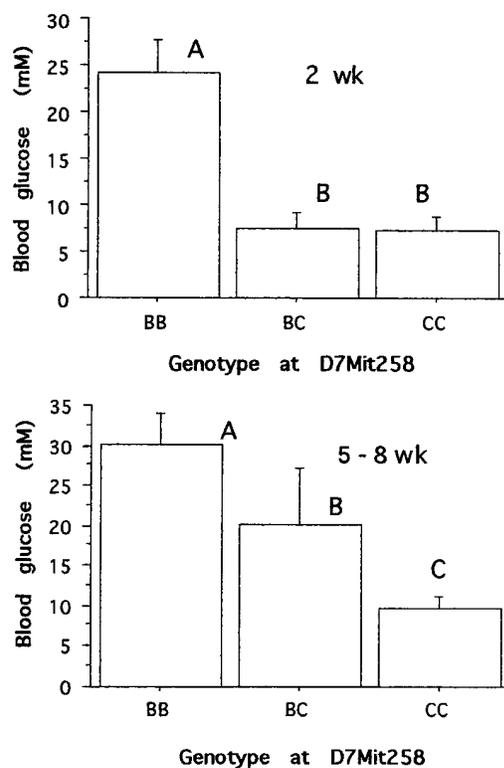


Figure 1. The three phenotypes in F2 progeny. The number of mice that developed hyperglycemia at 2 wk of age was 22 (9 males and 13 females). The number of mice that developed diabetes between 5 and 8 wk was 55 (23 males and 32 females). The nonaffected mice numbered 41 (25 males and 16 females). These mice were retrospectively classified based on the time course of elevation of blood glucose. This classification was in agreement with genotypes at the closely linked microsatellite marker to *Mody*, D7Mit258: BB type developed diabetes at 2 wk, BC type developed diabetes between 5 and 8 wk, and CC type did not develop diabetes. Data absent due to death of animals were replaced by the latest available data as described in Methods. Bars, 1 SD. Letters A, B, and C represent results of ANOVA. The different letters indicate difference at $P < 0.01$.

2 wk of age were tightly linked with D7Mit258, with a peak LOD score of 49.7. However, mean blood glucose levels between 5 and 8 wk of age showed linkage with D7Mit258, but with a lower but still significant LOD score of 28.2 (Fig. 2).

The assumption was then made that the 22 early-onset diabetic phenotype mice were homozygous for the mutant *Mody* gene, the 55 mice that developed diabetes between 5 and 8 wk of age were heterozygotes, and the 41 mice that did not develop diabetes had the wild-type gene. Using this qualitative genotype assignment, we searched for the position of the *Mody* locus among the seven microsatellite markers. As shown in Table I, the disease locus was found to be tightly linked to D7Mit258 (LOD score of 71.0). The absence of any recombination indicates that there was complete segregation of the qualitative disease phenotype and D7Mit258. Since there was such close proximity, we used genotypes at D7Mit258 as the surrogate genotype of the *Mody* locus for pathological investigations.

Pathological investigations. Blood glucose levels were slightly higher in homozygotes (BB type) than in the other genotypes in 1-d-old mice (Table II). On day 14, blood glucose

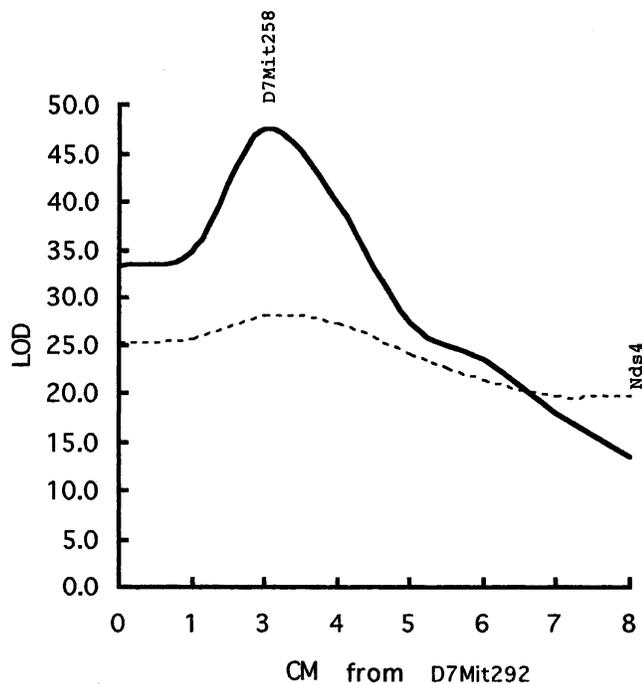


Figure 2. QTL analyses using two independent blood glucose measurements. The LOD score threshold using a set of 1,000 permutation traits was found to be 4.8 ($P < 0.001$), indicating all seven markers were significantly linked to blood glucose levels. *Solid line*, QTL analysis using blood glucose levels at 2 wk of age from 118 F2 mice. *Broken line*, QTL analysis using mean blood glucose levels between 5 and 8 wk of age.

levels were much higher in homozygotes than in the other two genotypes. There were no gender differences in blood glucose levels or other cytometric parameters at both 1 and 14 d of age.

Pancreatic samples for each gender and for each age were selected for morphological analysis after determination of genotype. Since these morphometric data did not show any gender-specific changes, male and female data were pooled. The islet areas in homozygotes were much smaller than those in the other two genotypes (Table II). Immunocytochemical examination revealed that homozygotes had a markedly lower density of active β cells than did either heterozygotes or wild-type mice within 24 h of delivery and at 2 wk of age (Fig. 3, and Table II). However, there was a striking increase in the density of α cells in the homozygotes. It is of note that heterozygotes also had significantly lowered densities of active β cells at 14 d of age.

Electron microscopy of the pancreatic tissues revealed that the islets of wild-type mice had abundant secretory cells with mature granules on the day of birth (Fig. 4, A and B). At 2 wk of age, granules had increased in density and quantity (Fig. 5, A and B). In most β cells, the rough endoplasmic reticulum was inconspicuous, and the Golgi complexes were small (Fig. 4 B and Fig. 5 B). The mitochondria were elongated or circular, slender, and filled with numerous closely packed mitochondrial cristae (Fig. 4 B and Fig. 5 B). The α cells were characterized by a peripheral location, larger and more electron-dense secretory cell granules than the β cell granules, and containment within tightly fitting smooth membranous sacs. There was a large amount of rough endoplasmic reticulum organized into compact parallel, tubular vesicles. Similarly, the Golgi

Table II. Pancreatic Morphometric Parameters in Mice of the Three Genotypes

Parameters	Genotype at D7Mit258		
	BB	BC	CC
On the day of birth			
No. of mice	18	47	26
Blood glucose (mM)	5.7 \pm 2.9 ^A	3.9 \pm 1.7 ^B	3.1 \pm 1.1 ^B
Body weight (g)	1.1 \pm 0.3 ^A	1.3 \pm 0.2 ^A	1.3 \pm 0.2 ^A
No. of examined mice			
Relative islet area (%)	1.7 \pm 1.0 ^A	4.1 \pm 0.2 ^B	5.6 \pm 2.5 ^B
Insulin-positive area in the islet (%)	11.1 \pm 3.0 ^A	49.6 \pm 12.0 ^B	45.6 \pm 4.2 ^B
Glucagon-positive area in the islet (%)	51.2 \pm 8.8 ^A	11.5 \pm 6.4 ^B	13.8 \pm 8.1 ^B
At 14 d of age			
No. of mice	16	41	20
Blood glucose (mM)	24.8 \pm 4.3 ^A	8.2 \pm 2.4 ^B	7.6 \pm 1.3 ^B
Body weight (g)	6.3 \pm 1.3 ^A	6.9 \pm 1.5 ^B	7.6 \pm 1.4 ^B
No. of examined mice			
Relative islet area (%)	2.0 \pm 0.7 ^A	4.5 \pm 0.6 ^B	4.3 \pm 1.1 ^B
Insulin-positive area in the islet (%)	10.8 \pm 1.8 ^A	45.4 \pm 7.4 ^B	56.2 \pm 3.2 ^C
Glucagon-positive area in the islet (%)	41.4 \pm 12.7 ^A	13.7 \pm 6.9 ^B	15.2 \pm 4.1 ^B

Values are mean \pm SD. Means of differences were compared by one-way ANOVA. Statistically significant differences between means were evaluated by Scheffe's test, which was applied when one-way ANOVA indicated significant differences ($P < 0.05$). Means in one row with a different superscript are significantly different ($P < 0.05$).

complex was larger and better organized than its counterpart in β cells (data not shown).

In heterozygous mice, the amount of secretory granules was comparable to that of wild-type mice on the day of birth. However, at this stage, early signs of accelerated insulin production were detectable, as evidenced by a slight swelling of mitochondria and increased amounts of endoplasmic reticulum and Golgi complexes (Fig. 4, C and D). At 2 wk of age, the β cell structure revealed adaptive responses (Fig. 5, C and D): there was a large amount of rough endoplasmic reticulum, and numerous tubular vesicular shapes became prominent. The Golgi complex was enlarged, and its components were more dispersed throughout the cytoplasm. Mitochondria were numerous and had increased in size, and secretory cells showed partial degranulation.

In contrast to the heterozygotes, homozygotes did not show such marked changes at prehyperglycemic stages on the day of birth (Fig. 4, E and F). The Golgi complexes were not enlarged, nor were their components more dispersed throughout the cytoplasm. Mitochondria were slender, and their fine structures remained intact, although there was a greater amount of rough endoplasmic reticulum. The β cell granules were much sparser in quantity and smaller than in the controls. In addition, the cytoplasm of β cells was smaller in area than in the other two genotypes. The decreased density of insulin secretory granules was in accord with findings made by immunohistochemistry that the density of active insulin-producing β cells

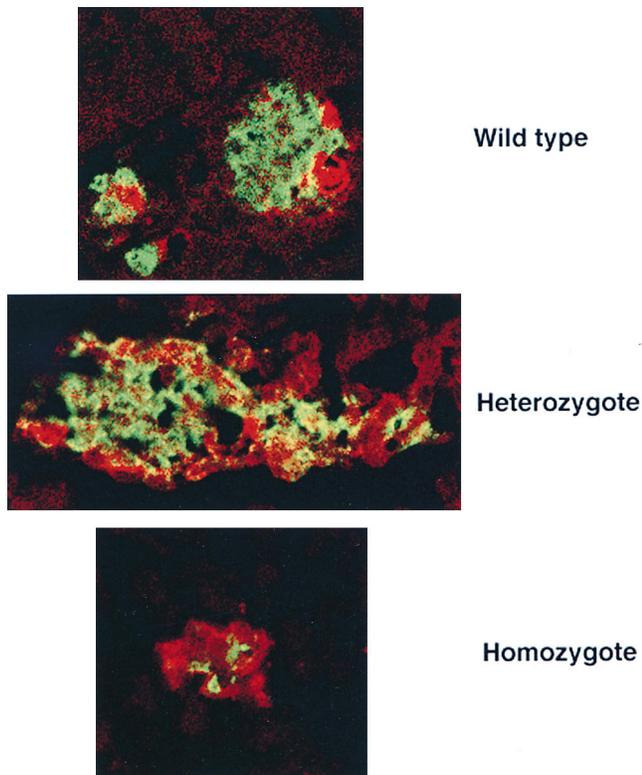


Figure 3. Islets of F2 mice on the day of birth. *Green*, Insulin-positive cells, indicating active β cells. *Red*, Glucagon-positive cells, indicating active α cells. There is a lower density of β cells and a higher density of α cells in the islets of F2 homozygous (BB) mice.

was much smaller in the homozygotes than in the other two genotypes. In contrast, α cells appeared to be intact (data not shown). At 2 wk of age (Fig. 5, *E* and *F*), α cell dominance persisted, and the cells appeared to be intact. In contrast, β cells had few insulin granules. In addition, the size of the granules was smaller than granules in wild-type animals. Mitochondrial swelling and a marked increase in the amount of endoplasmic reticulum were apparent by 2 wk of age.

Insulin and glucagon contents. Although an attempt was made to evaluate insulin content per islet and secretory function in vitro using islets, we were unsuccessful due to technical difficulties: islets from neonates of BB and BC types were too small and fragile. Therefore, we determined insulin and glucagon contents in the whole pancreas. Since both insulin and glucagon contents per pancreas did not show gender differences, male and female data were pooled. The presence of the mutant *Mody* gene appeared to decrease the insulin content of the pancreas dose-dependently, and this was evident even at birth: in homozygotes, insulin content was $\sim 1/7$ that of the CC genotypes, and in heterozygotes, it was $\sim 1/3$ that of the CC genotypes (Table III). In contrast, there was no difference in glucagon content between these groups. These decreases in insulin were underscored when the insulin contents were standardized relative to glucagon content: the insulin to glucagon ratios were 0.21 for BB genotypes and 0.42 for BC genotypes but 1.17 for CC genotypes.

At 14 d of age, the pancreases of all three genotypes appeared to be comparable in size as judged by organ weight. However, there was a remarkably disproportionate change in

the insulin to glucagon content ratio in BB genotypes (0.01). This insulin to glucagon ratio was significantly smaller than that of the other two genotypes (0.54 for BC and 1.11 for CC).

Gene and genotype frequencies at D7Mit258 in the F2 generation. 230 F2 progeny (112 males and 118 females) were examined to investigate a possible deviation from Mendelian inheritance. The neonates were killed within 24 h after delivery. Gene frequencies of the B allele at D7Mit258 were found to be equal to the expected value (218 of 460 meioses). The genotypes of 230 F2 progeny did not deviate significantly from the values expected from normal Mendelian inheritance: 46 (20%) for BB, 126 (55%) for BC, and 58 (25%) for CC genotypes.

Discussion

In this study, we confirmed our previous assignment of the *Mody* locus to the telomeric region of chromosome 7. QTL analysis using the blood glucose levels of F2 mice derived from B6 and Castaneus parents at 2 wk of age yielded one locus with a peak LOD score of 49.7 at D7Mit258. QTL analysis of mean blood glucose levels between 5 and 8 wk also yielded one peak at D7Mit258, with an LOD score of 28.2, although the peak was blurred. We observed three phenotypes in the F2 generation: a very early (2 wk) diabetic phenotype, a phenotype characterized by onset between 5 and 8 wk, and an unaffected phenotype. Assuming that the three phenotypes correspond to a homozygous, heterozygous, and wild-type state, respectively, then the mutant *Mody* was proven to be linked with D7Mit258, with a very high LOD score of 71.0. There-

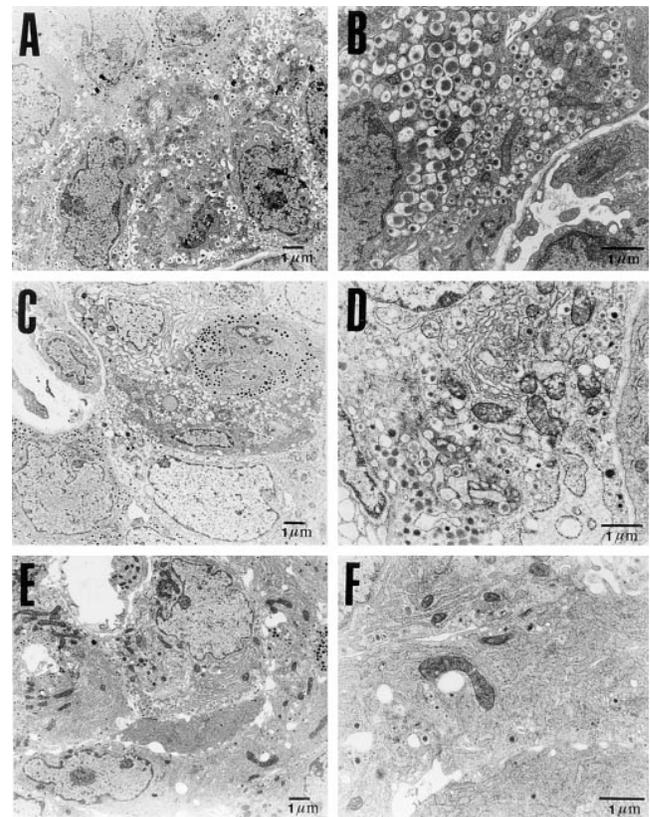


Figure 4. Electron micrographs of islets of F2 mice on the day of birth in wild-type (CC, *A* and *B*), heterozygous (BC, *C* and *D*), and homozygous (BB, *E* and *F*) genotypes. Bar, 1 μ m.

Table III. Insulin Contents and Insulin to Glucagon Ratios in the Pancreas of *Mody* Homozygotes at Birth and at 14 d of Age

Parameters	Genotype at D7Mit258		
	BB	BC	CC
On the day of birth			
No. of mice	10	12	10
Blood glucose (mM)	4.3±2.1 ^A	5.3±1.6 ^A	4.1±2.5 ^A
Body weight (g)	1.2±0.2 ^A	1.3±0.2 ^A	1.5±0.3 ^A
Pancreas (mg)	6.6±2.5 ^A	10.1±1.8 ^B	9.3±2.2 ^B
Insulin (pmol/pancreas)	15.6 (2.6) ^A	32.4 (2.0) ^B	114.6 (1.6) ^C
Glucagon (pmol/pancreas)	72.4 (1.9) ^A	77.4 (1.5) ^A	97.7 (1.5) ^A
Insulin to glucagon ratio*	0.21 (1.93) ^A	0.42 (1.70) ^B	1.17 (1.79) ^C
At 14 d of age			
No. of mice	14	20	9
Blood glucose (mM)	26.5±5.4 ^A	8.9±4.1 ^B	6.9±1.2 ^B
Body weight (g)	7.9±1.8 ^A	8.6±1.9 ^A	9.3±2.5 ^A
Pancreas (mg)	40.0±12.5 ^A	35.4±8.42 ^A	39.0±13.0 ^A
Insulin (pmol/pancreas)	3.9 (2.7) ^A	177.8 (1.9) ^B	361.4 (1.6) ^C
Glucagon (pmol/pancreas)	380.2 (1.5) ^A	329.6 (1.6) ^A	324.3 (1.3) ^A
Insulin to glucagon ratio*	0.01 (2.76) ^A	0.54 (1.68) ^B	1.11 (1.59) ^C

Values are mean±SD or geometrical mean (geometrical SD). Means of differences were compared by one-way ANOVA. Statistically significant differences between means were evaluated by Scheffe's test, which was applied when one-way ANOVA indicated significant differences ($P < 0.05$). Means in one row having different superscripts are significantly different ($P < 0.05$). *Insulin content divided by the glucagon content in the pancreas.

fore, we concluded that *Mody* was located at 72±3 cM on chromosome 7 near D7Mit258.

The regions of the *Mody* locus were syntenic to human 11p15.5 or 11q13.3. These areas include two diabetogenic genes, IDDM2 and IDDM4 (8–11). However, these genes are known to cause diabetes under the influence of IDDM1 (8–11), which causes an insulin-dependent diabetes unlike *Mody*. Therefore, we speculate that these loci are different from *Mody* because of the difference in function. We also showed that the *Mody* mutant gene followed normal Mendelian inheritance. Homozygous mice were neither diabetic nor small for the day of gestation at birth. These findings show a clear contrast to other diabetogenic genes reported in humans (12–17).

In this study, we could elucidate the pathophysiological consequences of *Mody*-associated diabetes during development. The high mortality of homozygotes of the *Mody* gene during the early neonatal period was very likely attributable to hyperglycemia. However, *Mody* homozygosity did not increase intrauterine fetal losses. Therefore, absence of wild-type *Mody* product during gestation is neither lethal nor teratogenic. On the other hand, the mutant *Mody* gene elicited unique morphological changes in β cells characterized by fewer insulin granules without apparent fetal hyperglycemia. This observation indicated that these changes are a direct result of the mutant *Mody* and are not due to physiological responses to hyperglycemia often affecting β cell morphology

after long-term hyperglycemia. In addition, morphometrical measurements clearly showed a low density of β cells with reciprocal increases in α cells in homozygous mice. Finally, we found that the *Mody* gene specifically decreased insulin but not glucagon content per pancreas during the perinatal period in a dose-dependent manner. The dramatic decrease in insulin content at 2 wk of age in homozygotes is concluded to be responsible for diabetes. The results clearly demonstrated a specific decrease in insulin content during the perinatal period in homozygotes. These would be corroborative for findings made by cytometry and electron microscopy. Thus, we concluded that the *Mody* gene is likely to be involved in both proliferation and insulin production in β cells.

The *Mody* homozygotes developed neonatal diabetes which had similar clinical features to permanent neonatal diabetes in humans (18–21). Permanent neonatal diabetes is a very rare disease, with an incidence of two to three cases per million deliveries. It is of note that in these cases, there is often a family history of diabetes. Neonatal diabetes is commonly accompanied by various degrees of anomaly, ranging from, in the most severe form, pancreatic agenesis to β cell agenesis in a milder form.

There are many studies that demonstrate that various transcriptional factors are involved in the development and maturation of the pancreas. It is of interest that the *Pax4* gene-deficient knockout mice show β cell agenesis (22). In these knockout mice, the islets completely lack β cells but have an abundance of α cells, thus exhibiting quite similar pathological alterations to those found in *Mody* homozygotes. Another gene of interest is *PDX1 (IPF1)*. The homozygous condition of

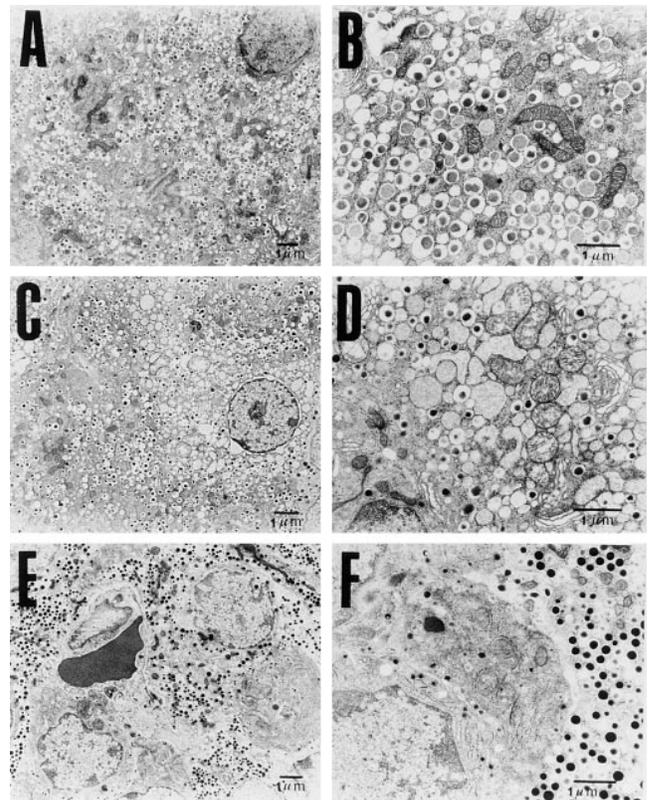


Figure 5. Electron micrographs of islets of F2 mice at 2 wk of age in wild-type (CC, A and B), heterozygous (BC, C and D), and homozygous (BB, E and F) genotypes. Bar, 1 μ m.

the *PDX1* (*IPF1*)-deficient gene, which results in pancreatic agenesis in both humans (21) and mice (23), has been proven to be associated with the hereditary diabetes *MODY4* in humans (6). Although the present murine model has some clinical characteristics in common with human *MODY*, it is premature to claim that our model is the murine equivalent of human *MODY* or human neonatal diabetes. Nevertheless, based on the similarities of the anomalies associated with the human genes, *IPF1* and *Pax4*, and *Mody*, we are tempted to speculate that *Mody* is involved in a hierarchy of developmental cascades of transcriptional factors.

One might argue that changes in β cell mass were not in parallel with the changes in insulin content. As a matter of fact, in heterozygotes, the decrease in insulin content occurs as early as birth, but the lowered β cell densities were not remarkable until 2 wk of age. Furthermore, in homozygotes, decreases in insulin content occurred without changing β cell mass after birth. That the *Mody* gene affects both insulin production and the number or density of β cells, two completely different phenotypic expressions, raises questions of how both phenotypes may be influenced by a single gene mutation, which effect is more primary, and which is crucial for diabetes. These questions are at present unanswered, because these two effects cannot be separated without the aid of positional cloning of the *Mody* gene.

One explanation might be to assume several pleiotropic effects of known or unknown factors. Of these, the assumption of insulin involvement, the levels of which changed dramatically during the early neonatal period, seemed attractive: embryonic insulin synthesis begins early in gestation, but fetal glucose levels are under the control of maternal glucose homeostasis. The embryonic insulin deficiency might cause deviation from normal β cell growth and differentiation by impairing the autocrine or paracrine pathway. However, this hypothesis is unlikely, because embryonic insulin appears to be unnecessary for β cell growth and differentiation (24). Thus, we should search for answers in another direction.

There is a growing body of evidence demonstrating that β cell replication and functional differentiation occur extensively in the perinatal period until weaning (25). Such qualitative and quantitative maturation of β cells is associated with adaptive processes to handle the oral glucose load after birth. During this period, influences of glucose dominate over other regulatory factors in β cell development. Thus, we are tempted to speculate *Mody* is involved in an as yet unidentified cascade, which is turned on by the initiation of oral glucose intake. The pleiotropic effects of the *Mody* gene appear to have versatile effects and may be involved in several different cascades. Obviously, further studies are necessary to clarify these issues.

In conclusion, this study strongly suggests a possibility that *Mody* is involved in β cell and islet development. In an effort to explain the possible pleiotropic effects of *Mody*, positional cloning and isolation of the *Mody* gene are currently ongoing projects in our laboratory.

Acknowledgments

This project was supported by grants-in-aid from the Ministry of Education, Culture and Sports of Japan (09470100, 09877073, and 09770232), and by a grant from the Japan Foundation for Aging and Health (1996 and 1997).

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