

Pathophysiological and Genetic Characterization of the Major Diabetes Locus in GK Rats

Joakim Galli, Hossein Fakhrai-Rad, Ashraf Kamel, Claude Marcus, Svante Norgren, and Holger Luthman

Genetic studies of the type 2 diabetes-like GK rat have revealed several susceptibility loci for the compound diabetes phenotype. Congenic strains were established for *Niddm1*, the major quantitative trait locus (QTL) for postprandial glucose levels, by transfer of GK alleles onto the genome of the normoglycemic F344 rat. Despite the polygenic nature of diabetes in GK, the locus-specific diabetes phenotype was retained in the congenic strain Niddm1a, containing a GK-derived genomic fragment of 52 cM from the *Niddm1* locus. Furthermore, *Niddm1* was divided into two non-overlapping loci, physically separated in the two congenic strains Niddm1b and Niddm1i with distinct metabolic phenotypes. Both strains displayed postprandial hyperglycemia and reduced insulin action in isolated adipose cells. Furthermore, Niddm1i already exhibits a pronounced in vivo insulin secretion defect at 65 days, while Niddm1b develops a relative insulin secretory defect at 95 days. This suggests that *Niddm1i* impairs mechanisms common to insulin secretion in pancreatic B-cells and insulin action in adipocytes. Niddm1b rats show signs of increasing insulin resistance with age associated with obesity, hyperinsulinemia, and dyslipidemia. Moreover, the data indicated nonallelic interaction (epistasis) between *Niddm1b* and *Niddm1i* on the postprandial glucose levels. These data emphasize the pathophysiological complexity of diabetes, even within an apparently single QTL, and demonstrate the potential of the GK model in transforming the multifactorial diabetes phenotype into single traits, suitable for positional cloning. *Diabetes* 48:2463-2470, 1999

Type 2 diabetes develops as a consequence of inadequate insulin secretion in subjects with insulin resistance (1). However, the molecular mechanisms involved in disease pathways that may lead to type 2 diabetes are just beginning to be outlined. Numerous family and twin studies have demonstrated the critical influence of environmental factors as well as a sizable impact

of genetic factors for the risk of developing type 2 diabetes (2-7). Monogenic variants of diabetes with autosomal dominant mode of inheritance (maturity-onset diabetes of the young [MODY]) or mitochondrial inheritance of disease have been described in recent years at the molecular and clinical levels (8-13). However, the common forms of the disease appear to be multifactorial, influenced by both polygenic and environmental factors. Dissection by linkage analysis of multifactorial diseases such as type 2 diabetes is facilitated by studies of inbred animal models with inherited susceptibility to disease. Such dissections make it possible to localize essential genes involved in disease pathways without prior knowledge of their roles in the disease phenotype. This strategy has successfully revealed distinct loci for several components of the metabolic syndrome, e.g., hypertension, glucose intolerance, insulin secretion, body weight, insulin action, and dyslipidemia (14-24).

The Goto-Kakizaki (GK) rat is an extensively studied model for type 2 diabetes. The phenotype of GK is well characterized and exhibits several features typical of type 2 diabetes, such as fasting hyperglycemia, impaired secretion of insulin in response to glucose, and insulin resistance, as well as late complications, e.g., neuropathy and nephropathy (25,26). In the original genetic linkage analysis of the F2-intercross between GK and the normoglycemic F344 rat, four major quantitative trait loci (QTL) were identified with genome-wide significance as well as 10 minor QTLs that affect the segregation of diabetes and its associated phenotypes (17). The generation of congenic strains is a straightforward approach for both verifying susceptibility genes residing within a QTL and investigating their pathophysiological implications. If the phenotype is retained in the congenics, this method allows for genetic fine mapping so that positional cloning becomes feasible. Furthermore, physiological characterization of congenic strains and heterozygous backcross animals provides clues to the contribution of a single QTL to the pathophysiology of a complex phenotype before identification and cloning of the corresponding gene or genes.

As a first step toward cloning diabetes susceptibility genes in GK, we established a set of congenic strains by repeated backcrossing of GK onto the normoglycemic F344. Here we present the phenotypic characterization of strains homozygous or heterozygous for the major diabetes QTL, *Niddm1*, which explains 30% of the genetic variation of postprandial glucose levels in F2 progeny from the cross between GK and F344. We demonstrate that at least two diabetes susceptibility genes reside in *Niddm1* on rat chromosome 1, and that they have distinct and allele-dose dependent effects on phenotypes that characterize type 2 diabetes and the metabolic syndrome.

From the Karolinska Institute (J.G., H.F.-R., A.K., C.M., S.N., H.L.); the Department of Molecular Medicine (J.G., H.F.-R., H.L.), Karolinska Hospital; and the Department of Pediatrics (A.K., C.M., S.N.), Pediatric Endocrine Research Unit, Huddinge University Hospital, Stockholm, Sweden.

Address correspondence and reprint requests to Joakim Galli, Karolinska Institute, CMM, Department of Molecular Medicine, Karolinska Hospital, L602, S-171 76 Stockholm, Sweden. E-mail: jga@gen.ks.se.

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AUC, area under the curve; EC₅₀, the concentration producing 50% of the maximum effect; IPGTT, intraperitoneal glucose tolerance test; KRP, Krebs-Ringer phosphate; MODY, maturity-onset diabetes of the young; QTL, quantitative trait locus; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcriptase-polymerase chain reaction.

RESEARCH DESIGN AND METHODS

Rat strains. GK and F344 rats were obtained and bred as described (17). GK-derived genetic intervals were transferred onto the F344 background by 10 successive backcrosses (N10) followed by intercrosses between heterozygous animals to establish homozygous congenic strains. At each generation, genetic markers from the *Niddm1* region were used to verify the integrity of the GK-susceptibility haplotype.

Intraperitoneal glucose tolerance test. Intraperitoneal glucose tolerance tests (IPGTT) were performed on male rats of 65 and 95 days of age, essentially as described (17). The ambient room temperature was kept constant at 25°C. The animals were fasted for 6–7 h; blood glucose levels were measured after tail vein incision at 0 (baseline), 15, 30, 60, and 90 min after injection of 2.0 g glucose per kilogram of body weight; and serum immunoreactive insulin levels were determined at 0, 15, and 30 min. The serum insulin levels in Tables 1, 2, and 3 were determined with an ELISA for rat insulin (Merckodia, Uppsala, Sweden), as described by the manufacturer. The insulin values in $\mu\text{g/l}$ obtained from the ELISA analysis were converted to picomoles per liter by multiplying by a factor of 174. The area under the curve (AUC) was calculated according to the trapezoid rule from glucose measurements (millimoles per liter) at baseline, 15, 30, 60, and 90 min. The glucose and insulin values presented in Fig. 2 were standardized by division with the corresponding mean values for F344 and subsequently multiplied by the mean values for F344 presented in Table 2. The mean glucose values for F344 in the experiment presented in Fig. 2 were 5.0 (basal), 18.6 (15 min), 13.8 (30 min), 6.6 (60 min), and 6.2 mmol/l (90 min). The corresponding insulin mean values were: 63 (basal), 200 (15 min), and 215 pmol/l (30 min).

Lipid analysis. The serum levels of triglyceride, total cholesterol, and HDL cholesterol were determined with Vitros TRIG and Vitros CHOL slides (Johnson & Johnson, Rochester, NY), and liquid N-geneous HDL-c reagent kit (Biomed-RK, Jönköping, Sweden).

Lipogenesis and lipolysis. Male rats (75 days) were decapitated after carbon dioxide anesthesia, and the epididymal fat depots (1–2 g) were removed. Adipocytes were prepared as described (27). Studies of glucose incorporation into lipids (lipogenesis) were performed at a glucose concentration of 1 $\mu\text{mol/l}$, at which glucose transport into the cells is rate limiting (28). The adipocytes were incubated at 2% (vol/vol) concentration in 0.5 ml Krebs-Ringer phosphate (KRP) buffer containing 40 mg/ml albumin (Sigma, Stockholm, Sweden), 0.2 $\mu\text{mol/l}$ [^3H]glucose (5×10^6 cpm), 1.0 $\mu\text{mol/l}$ unlabeled glucose, and insulin at the indicated concentrations. At each insulin concentration, the analysis was performed in triplicate at 37°C for 2 h, and the reactions were terminated by rapid chilling to 4°C. Incorporation of glucose into lipids was determined as described (29). Briefly, 45 μl of 6.0 M H_2SO_4 and 4.0 ml of toluene with 2,5-diphenyloxazole were added to each vial and the vials were left at room temperature overnight before liquid scintillation counting. For characterization of lipolysis, adipocytes were incubated for 2 h at 37°C in KRP buffer containing 40 mg/ml albumin (Sigma) and 5.6 mmol/l glucose. The final adipocyte suspension was 1% (vol/vol). At the end of the incubation, an aliquot of the medium was removed for analysis of glycerol release (30), which was used as an index of lipolysis. To assess maximal lipolysis, noradrenaline (1 nmol/l to 0.1 mmol/l) was added to the incubation media (31). Lipogenesis and lipolysis were expressed per cell surface area in order to eliminate differences depending solely on adipocyte size (31). The maximal insulin-induced lipogenesis was calculated as the difference between glucose incorporation at maximum minus the incorporation of glucose in the absence of insulin. Maximal noradrenaline-induced stimulation of lipolysis (responsiveness) was calculated from each individual dose-response curve as the maximum glycerol release minus glycerol release in the absence of noradrenaline. The concentration of noradrenaline or insulin that produced 50% of the maximum effect (EC_{50} , sensitivity) was calculated from the individual dose-response curves.

Insulin mRNA analysis. The RNA levels of the rat insulin genes, *Ins1* and *Ins2*, in pancreas were determined by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Five-month-old male rats were fasted for 7 h and pancreases were isolated directly or after glucose challenge. In the latter case, glucose (2 g/kg and subsequently 1 g/kg body weight) was injected intraperitoneally at 0 and 60 min, and the rats were sacrificed at 120 min. Total pancreatic RNA (0.75 μg), prepared as previously described (32), was reverse-transcribed in a total volume of 20 μl , using BRL Superscript II (Life Technologies, Täby, Sweden), as described by the manufacturer. The two transcripts from *Ins1* and *Ins2* were reverse transcribed with a primer common to both insulin genes (5'-TTTATCA TTGACAGGGGT). The cDNA reaction (5 μl) was directly introduced into 25 μl PCR solution, containing Dynazyme DNA polymerase and buffer (Finnzymes OY, Espoo, Finland). The *Ins1* and *Ins2* genes were amplified in separate reactions with ^{32}P -labeled specific primers (*Ins1* primers: 5'-GTGACCAGCTACAATC ATAG and 5'-GTGCCAAGGCTGAAGATCC; *Ins2* primers: 5'-GTGACCAGCTAC AGTCGGAA and 5'-GTGCCAAGGCTGAAGGTCA) by denaturation at 94°C for 3 min, followed by 20 cycles consisting of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min, and a final extension for 7 min at 72°C. The insulin-specific products accu-

mulated exponentially up to cycle 24 (data not shown). The samples (15 μl) were separated on 6% polyacrylamide gels, the gels were dried, and the radioactivity visualized and quantified by phosphorimager analysis (Fujix BAS 1000; Fuji, Tokyo).

Genotype analysis and localization of markers. Rats were genotyped by PCR amplification of microsatellite markers essentially as previously described (15), with the exception that [$\gamma\text{-}^{32}\text{P}$]ATP was used to label one primer in each pair. For the genetic mapping of new markers, the 45 rats with the most extreme glucose values from our first F2 intercross were genotyped (17), and markers were placed on the genetic map using the computer package Mapmaker/exp 3.0 (33).

Generation of new restriction fragment length polymorphism markers and Southern blot analysis. The hybridization probes were synthesized by RT-PCR or genomic PCR, using available rat cDNA sequences and gene-specific primers (34–36). Total RNA was prepared as previously described (32). A total of 6 μg of RNA was transcribed using BRL Superscript II (Life Technologies), as described by the manufacturer. For the *Jak2* probe, total RNA prepared from the whole body of a 1-day old rat was used in the reverse transcriptase reaction (cDNA primer: 5'-AAGGGCCCGTGACACGAG) and 2 μl of the reverse transcriptase reaction was introduced in the subsequent PCR amplification (primers: 5'-AAGGGCC CGTGACACGAG and 5'-GAAGAGCAAAGCCCACCTG, PCR-profile: 96°C for 4 min, followed by 35 cycles consisting of 96°C for 30 s, 55°C for 1 min, 72°C for 2 min, and a final extension for 7 min at 72°C). The *Jak2* gene was mapped by a *HindIII* restriction fragment length polymorphism (RFLP) with fragment lengths of 8.6 kb in GK and 6.4 kb in F344. *Pnlp* mRNA from total pancreatic RNA was reverse transcribed using primer: 5'-ACTACAGAAGTTGAACACTCTG. PCR conditions were identical as for the *Jak2* reaction, with the exception of an annealing temperature of 50°C (primers: 5'-CGATGCCAGTTTGTGGATG and 5'-ACT ACAGAAGTTGAACACTCTG). A total of 1 μl from the first amplification was used as template in a second nested PCR (primers: 5'-ACTTAGGATTGGAATGAGC and 5'-TTGGGTAGAGTTGGTTGAT; conditions were as for *Jak2*, except that annealing was performed at 53°C). A *StuI* RFLP was used to genetically map the *Pnlp* gene: GK (18 kb) and F344 (14 and 4 kb). The *Htr7* gene was amplified by genomic PCR at the same conditions as for *Pnlp* (primers for first PCR amplification: 5'-CGAAATCATTGGCTGAGACTG and 5'-GGGTACTCTTCTGAACT-GTGG; second nested PCR with primers: 5'-TGGCTTCTGCTTCTTCTTGG and 5'-CTGCTTCTTACCTGTCTTA). An *MspI* RFLP was identified for *Pnlp* that generated fragments of 5.5 kb for GK and 4.5 kb for F344. Southern blot analysis was performed as described (37). High molecular weight DNA was extracted from rat liver, and genomic DNA (10 μg) was digested with the appropriate restriction endonuclease, size fractionated in 0.8% agarose gels, and transferred to nylon membranes (Zeta-probe; Bio-Rad, Hercules, CA). The RFLP probes were ^{32}P -labeled by random priming (38).

RESULTS

Establishment of Niddm1 congenics. A breeding protocol was established to allow for the transfer of the GK-*Niddm1* diabetes susceptibility allele onto the background genome of the normoglycemic F344 rat. A long interval was transferred from GK to F344 to assure that no susceptibility genes in this chromosomal region were lost (Fig. 1). The GK-specific region in the congenic strain F344.GK-Niddm1a (*Niddm1a*) was 52 cM long and contained the complete 20 cM 95% CI previously defined for *Niddm1* flanked by ~15 cM of additional GK alleles (17). We produced a number of sub-strains from *Niddm1a* to define the location of the *Niddm1* susceptibility gene/genes. Two of these strains, F344.GK-Niddm1b (*Niddm1b*) and F344.GK-Niddm1i (*Niddm1i*), retain 28 and 22 cM of the GK interval. The GK regions in *Niddm1b* and *Niddm1i* are distinct and nonoverlapping since two markers (*Cyp2c12* and *D1Mgh29*), separating the two GK regions, are homozygous for F344 alleles (Fig. 1). All congenic strains were passed through 10 successive generations of backcrossing (on the average only 0.1% GK material remains in the congenics). Backcrossing was designed to introduce mitochondrial DNA and sex chromosomes only originating from F344. To verify the purity of the strains, we performed a genome-wide analysis with 111 markers spaced at an average of 20 cM. Special care was taken to analyze known loci for diabetes associated phenotypes (17,18). No remaining GK-derived alleles were found.

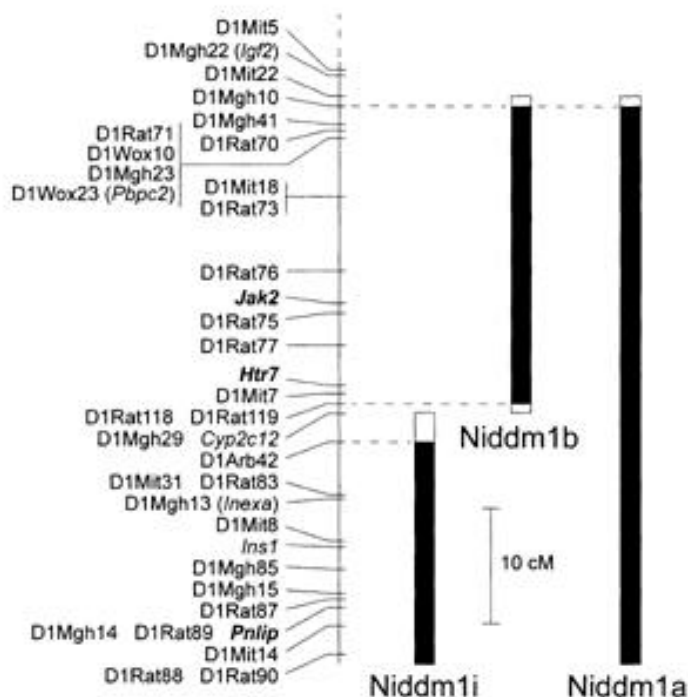


FIG. 1. Genetic map of the distal part of rat chromosome 1 and extension of the congenic strains. The extent of GK-derived genomic intervals is displayed as black bars for the three congenic strains: Niddm1a, Niddm1b, and Niddm1i. White bars indicate genomic intervals spanning the crossover points between GK and F344 derived alleles, as defined by the closest flanking markers. The GK-derived chromosome regions are 52 ± 3 cM in Niddm1a, 28 ± 1 cM in Niddm1b, and 22 ± 1 cM in Niddm1i.

Phenotypic characterization of Niddm1 subloci. The intraperitoneal glucose tolerance test (IPGTT) was used to identify the *Niddm1* locus in the original F2-intercross (17). This phenotyping method was also applied to characterize the congenic strains. However, to further challenge the animals, the IPGTT was performed in older rats (95 vs. 70 days). No sex-specific effect for *Niddm1* was observed in our original genetic analysis of the GK rat (17). We therefore only included male rats in this study. The larger size of the males in comparison with females facilitated the experimental procedure, resulting in less experimental variation. Niddm1a male rats with the complete *Niddm1* chromosome region (52 cM) differed significantly from F344 rats in glucose tolerance during IPGTT (Fig. 2A). The most pronounced difference was observed at 15 min after glucose injection when the mean glucose concentration in Niddm1a was 4.0 mmol/l (26%) higher than in F344 ($P = 0.0005$). Also, the two congenic strains carrying separate parts of the *Niddm1* locus displayed significantly higher postprandial glucose concentrations compared with control F344 rats. The results of the IPGTT of Niddm1b and Niddm1i compared with the control F344 rat are shown in Fig. 2B. At 15 min after glucose injection, Niddm1b and Niddm1i exhibited 2.3 mmol/l (15%) and 4.7 mmol/l (31%) higher glucose levels than F344 ($P = 0.008$ and $P = 0.00005$). Furthermore, the data indicate that nonallelic interaction (epistasis) is operating within the *Niddm1* locus. The AUC of F344 was subtracted from that obtained from each congenic strain and the AUC increases (over that in F344) of the two

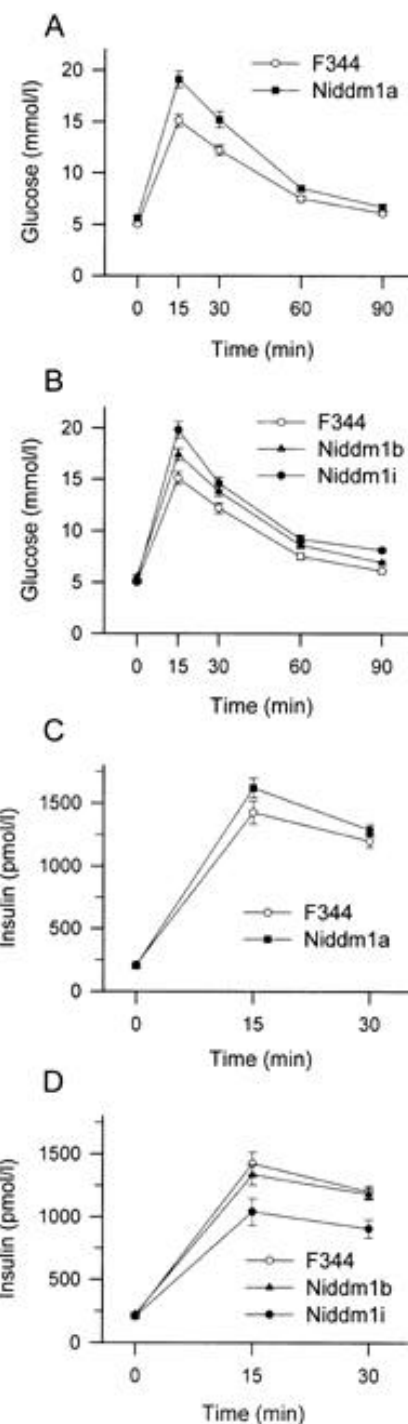


FIG. 2. IPGTT results for *Niddm1* congenic strains and F344. Male rats (95 days) from strains Niddm1a ($n = 11$), Niddm1b ($n = 17$), Niddm1i ($n = 12$), and F344 ($n = 20$) were subjected to IPGTT. After glucose injection the concentrations of blood glucose (A and B) and serum insulin (C and D) were determined at the indicated time points. As compared with F344, the glucose AUC was significantly higher in Niddm1a ($P = 0.0007$), Niddm1b ($P = 0.002$), and Niddm1i ($P = 0.00001$). The serum insulin levels at 15 and 30 min were significantly lower in Niddm1i than in F344 ($P = 0.01$ and 0.002). No differences in body weight were observed in this experiment, when comparing Niddm1a, Niddm1b, or Niddm1i with F344. The values presented here were normalized to the F344 values in the second experiment on 95-day-old rats (Table 2), as described in RESEARCH DESIGN AND METHODS. To eliminate the effects of unpredictable environmental factors resulting in differences between experiments, we included F344 control animals in each experimental series. Results are shown as mean \pm SE.

TABLE 1
Diabetes-associated phenotypes in Niddm1 congenic strains and F344 at age 65 days

Phenotype	F344	Niddm1b/F344	Niddm1b	Niddm1i/F344	Niddm1i
<i>n</i>	15	12	11	8	11
Weight (g)	207 ± 3	216 ± 4	228 ± 4‡	202 ± 4	197 ± 5
Glucose (mmol/l)					
0 min	4.8 ± 0.1	4.6 ± 0.1	4.8 ± 0.1	4.6 ± 0.2	4.7 ± 0.1
15 min	15.9 ± 0.4	17.0 ± 0.5	17.3 ± 0.6*	15.4 ± 1.0	17.2 ± 0.5*
30 min	9.4 ± 0.3	10.3 ± 0.4	10.6 ± 0.5*	8.2 ± 0.7	10.5 ± 0.4*
60 min	4.9 ± 0.2	5.2 ± 0.3	5.5 ± 0.2	6.0 ± 0.1†	4.9 ± 0.1
90 min	4.8 ± 0.2	5.2 ± 0.2	5.1 ± 0.1	5.6 ± 0.2*	4.8 ± 0.2
AUC	705 ± 12	755 ± 18*	774 ± 20†	712 ± 26	748 ± 18
Insulin (pmol/l)					
0 min	77 ± 7	109 ± 9†	122 ± 22*	78 ± 10	62 ± 18
15 min	1,234 ± 182	1,259 ± 178	1,263 ± 143	1,111 ± 163	542 ± 76†
30 min	498 ± 68	857 ± 64†	980 ± 153†	351 ± 74	398 ± 68

Data are means ± SE. Each congenic strain was compared with F344 (Student's *t* test). **P* < 0.05, †*P* < 0.01, ‡*P* < 0.001.

substrains (Niddm1b and Niddm1i) were added up. The sum of the AUC increases for the two substrains (325) was distinctly larger than the AUC increase (171) of the parental strain (Niddm1a), clearly indicating epistasis.

A discriminating feature of Niddm1i in comparison with Niddm1b was the significantly lower serum insulin levels at 15 and 30 min (*P* = 0.03 and *P* = 0.002). At 15 and 30 min postinjection the insulin values in Niddm1i were 385 pmol/l (27%, *P* = 0.012) and 294 pmol/l (24%, *P* = 0.002) lower than in F344 (Fig. 2D). No significant differences in insulin levels were observed when comparing either Niddm1a or Niddm1b with F344 (Fig. 2C and D). We conclude that the *Niddm1* locus contains at least two separate genes affecting glucose homeostasis, since the *Niddm1i* and *Niddm1b* both affect glucose levels but cover different parts of chromosome 1 and display major differences in glucose-stimulated insulin secretion in vivo.

Diabetes development in Niddm1b and Niddm1i. To further investigate the diabetes phenotype associated with the GK alleles at the *Niddm1b* and *Niddm1i* loci, congenic rats were studied at different ages in a prospective study. This study, independent of the experiment presented in Fig. 2, was also performed to confirm the results of the initial experiment. In order to characterize the phenotypic effects of each GK-allele at the loci, also GK/F344 heterozygous animals were studied. The heterozygous animals were produced by backcrossing of Niddm1b or Niddm1i to F344. These animals were denoted Niddm1b/F344 and Niddm1i/F344, to indicate the heterozygous nature at each locus. Male rats carrying the *Niddm1b* or *Niddm1i* locus in homozygous (GK/GK) or heterozygous (GK/F344) form and F344 rats were subjected to IPGTT at 65 and 95 days of age. At 185 days of age, the basal levels of blood glucose, serum insulin, triglyceride, total cholesterol, and HDL cholesterol were determined; the animals were then killed and the epididymal fat depots weighed.

At 65 days of age, Niddm1b and Niddm1b/F344 showed slightly elevated postprandial glucose levels at the early time points (15 and 30 min) during the IPGTT, compared with F344 rats (Table 1). However, the serum insulin levels (basal and 30 min) were significantly higher in Niddm1b and Niddm1b/F344 (Table 1). The basal insulin levels in Niddm1b and Niddm1b/F344 were 58 and 42% higher compared with F344,

and at 30 min postinjection the corresponding increases were 97 and 72%. In concordance with the experiment shown in Fig. 2, postprandial glucose levels in mid-aged (95 days) Niddm1b rats were significantly higher than in F344 (Table 2). No difference in glucose levels was observed between Niddm1b/F344 and F344. At this age the serum insulin levels in the heterozygous animals were still slightly higher (15 and 30 min). In contrast, a slight insulin decrease was observed in the homozygous animals (Table 2). Although the insulin levels in Niddm1b were not significantly different from F344 during the IPGTT, the insulin secretion was obviously impaired in the light of the increased glucose levels.

Later in life (185 days), both basal glucose and basal insulin levels in Niddm1b were significantly higher than in F344 rats (Table 3). The levels of triglyceride and HDL cholesterol were also significantly higher in Niddm1b than in F344 rats (Table 3), while the total cholesterol levels were not different. In contrast to the cholesterol levels in Niddm1b rats, both total and HDL cholesterol levels in the heterozygous rats (Niddm1b/F344) were significantly lower than in F344 rats. No differences in basal glucose, insulin, or triglyceride levels were found between Niddm1b/F344 and F344. Moreover, the Niddm1b rats were significantly heavier (10, 9, and 6% at 65, 95, and 185 days) than F344 rats in this experimental series (Tables 1–3), and the epididymal fat weight was increased by 18% (Table 3). The increase in Niddm1b body weight was not observed in the first experiment (data not shown), and we cannot exclude the possibility that this increase reflects merely a consequence of differences in nutrition during early life. However, genetic linkage to body weight was observed in the original genetic analysis of the GK rat in the region corresponding to *Niddm1b* (17).

In Niddm1i the postprandial glucose levels at 95 days were, as expected, significantly higher compared with F344 (Table 2). Furthermore, similar to the first experimental series (Fig. 2), the serum insulin levels during the IPGTT were lower in Niddm1i rats (Table 2). Also in 65-day-old Niddm1i rats the insulin levels were lower compared with F344, indicating a pronounced and early B-cell defect in Niddm1i. At 15 min post-glucose injection the insulin levels in Niddm1i were 56% of those in F344, despite slightly elevated blood glucose levels (Table 1). No major differences in glu-

TABLE 2
Diabetes-associated phenotypes in Niddm1 congenic strains and F344 at age 95 days

Phenotype	F344	Niddm1b/F344	Niddm1b	Niddm1i/F344	Niddm1i
<i>n</i>	15	12	11	8	11
Weight (g)	279 ± 4	280 ± 4	305 ± 5‡	275 ± 4	270 ± 6
Glucose (mmol/l)					
0 min	5.1 ± 0.1	4.9 ± 0.1	5.6 ± 0.3	5.3 ± 0.1	5.8 ± 0.2†
15 min	15.1 ± 0.5	15.4 ± 0.6	17.4 ± 0.7†	16.6 ± 1.1	18.5 ± 0.8‡
30 min	12.2 ± 0.3	12.2 ± 0.5	14.1 ± 0.7†	13.1 ± 0.4	14.0 ± 0.4‡
60 min	7.5 ± 0.3	7.2 ± 0.3	8.1 ± 0.5	7.3 ± 0.3	8.5 ± 0.3*
90 min	6.1 ± 0.2	6.0 ± 0.2	7.1 ± 0.2†	6.6 ± 0.2	7.2 ± 0.2‡
AUC	855 ± 21	846 ± 29	971 ± 35†	901 ± 31	1,001 ± 28‡
Insulin (pmol/l)					
0 min	210 ± 27	208 ± 23	238 ± 80	260 ± 38	225 ± 48
15min	1,425 ± 205	1,589 ± 141	1,166 ± 287	1,787 ± 142	810 ± 193*
30 min	1,200 ± 186	1,507 ± 138	1,141 ± 297	1,563 ± 168	792 ± 189

Data are means ± SE. Each congenic strain was compared with F344 (Student's *t* test). **P* < 0.05, †*P* < 0.01, ‡*P* < 0.001.

cose or insulin levels were found between Niddm1i/F344 and F344 rats at 65 or 95 days. At the age of 185 days, both Niddm1i and Niddm1i/F344 displayed higher HDL cholesterol levels (Table 3).

Insulin action in adipocytes. To characterize further the *Niddm1* phenotype, we investigated adipocytes isolated from the epididymal fat depot of rats at age 75 days (Niddm1i, Niddm1b, F344, and GK). Lipogenesis was determined as incorporation of radioactive glucose into lipids in response to increasing concentrations of insulin. Compared with F344, adipocytes from both the Niddm1b and Niddm1i had significantly lower basal and insulin-induced lipogenesis, but significantly higher than adipocytes from GK rats, which demonstrated severely reduced insulin action (Fig. 3). There was no significant difference between the two congenic strains (Niddm1b and Niddm1i). The EC₅₀ of insulin-induced lipogenesis revealed no interstrain difference in insulin sensitivity. In addition, lipolysis was studied by measuring glycerol release from isolated adipocytes. No significant differences were observed in either basal lipolysis or noradrenaline-induced lipolysis (data not shown). This result demonstrates that the observed differences in insulin action reflect a pathway-specific defect and not a general adipocyte dysfunction.

Candidate gene function and syntenic conservation. The rat has two functional insulin genes on chromosome 1. The insulin 1 gene (*Ins1*) is located in the GK interval con-

tained in Niddm1i and was an obvious candidate for mutations causing the impaired glucose homeostasis. The *Ins2* gene is located outside the GK regions in the congenic strains. We have previously reported a difference in the *Ins1* promoter sequence between GK and F344 rats at nucleotide position -258 bp relative to the transcription start site (no differences were found in the transcribed region of the gene), but a similar relative abundance of *Ins1* and *Ins2* mRNA in pancreas from both strains (17). To investigate a potential role of this genetic variation in more detail, the pancreatic levels of mRNA for *Ins1* and *Ins2* were estimated by semiquantitative RT-PCR in GK, F344, and Niddm1i rats after a fasting period of 7 h and after 2 h of repeated glucose injections (Fig. 4). Compared with F344, the total insulin (*Ins1* and *Ins2*) mRNA level was 30% higher in Niddm1i rats, despite the impaired insulin response demonstrated during IPGTT. However, the relative expression of *Ins1* and *Ins2* did not differ among the strains in either the basal or the glucose-stimulated state. Thus, we exclude *Ins1* as a likely candidate for the *Niddm1i* phenotype. The insulin RNA data show that the defect in insulin secretion observed in Niddm1i is located downstream of the regulation of insulin transcription.

Information concerning the corresponding homologous regions to *Niddm1* in humans and the mouse is important for locating candidate genes, and for comparisons of the *Niddm1* rat locus with other susceptibility loci linked to type 2 diabetes

TABLE 3
Diabetes-associated phenotypes in Niddm1 congenics and F344 at age 185 days

Phenotype	F344	Niddm1b/F344	Niddm1b	Niddm1i/F344	Niddm1i
<i>n</i>	9	11	10	8	10
Weight (g)	365 ± 9	354 ± 4	386 ± 4*	356 ± 4	350 ± 6
Basal glucose (mmol/l)	5.7 ± 0.1	5.6 ± 0.2	6.2 ± 0.1†	5.9 ± 0.1	5.7 ± 0.1
Basal insulin (pmol/l)	378 ± 71	423 ± 48	631 ± 38†	408 ± 47	472 ± 50
Fat weight (g)	10.5 ± 0.7	9.4 ± 0.2	12.4 ± 0.3*	9.8 ± 0.4	10.2 ± 0.4
Triglyceride (mmol/l)	2.30 ± 0.09	2.07 ± 0.13	3.03 ± 0.16†	2.25 ± 0.09	2.16 ± 0.14
Total cholesterol (mmol/l)	2.12 ± 0.05	1.87 ± 0.03‡	2.22 ± 0.05	2.18 ± 0.02	2.31 ± 0.08
HDL cholesterol (mmol/l)	0.96 ± 0.02	0.87 ± 0.02†	1.05 ± 0.03*	1.05 ± 0.02†	1.08 ± 0.04†

Data are means ± SE. Each congenic strain was compared with F344 (Student's *t* test). **P* < 0.05, †*P* < 0.01, ‡*P* < 0.001.

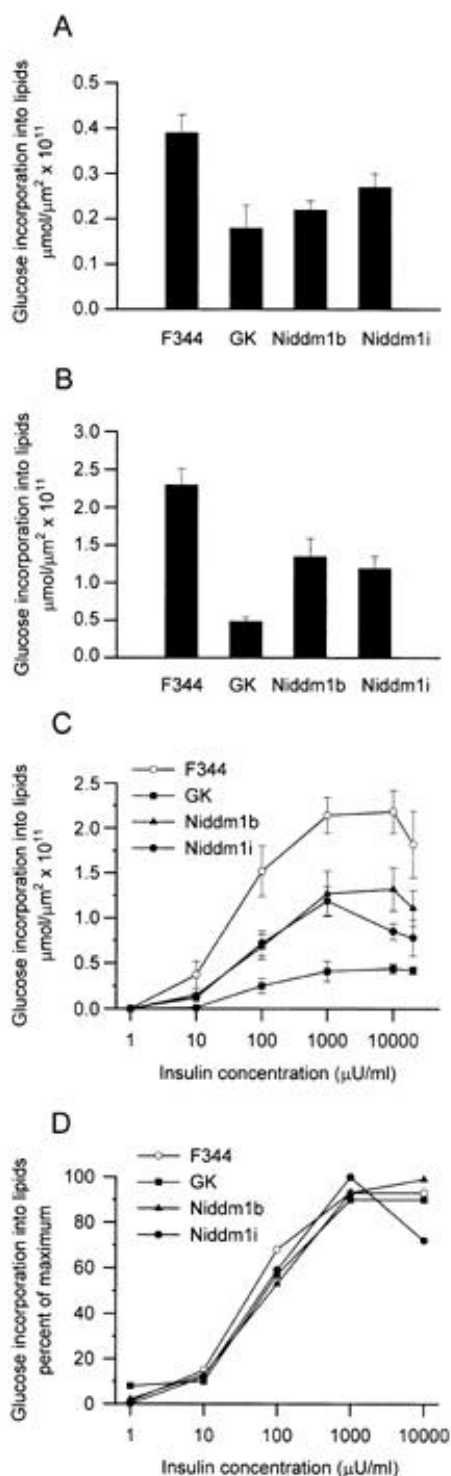


FIG. 3. Insulin-stimulated lipid synthesis (lipogenesis) in adipocytes isolated from epididymal fat. Adipocytes were isolated from two-month-old male F344 ($n = 7$), GK ($n = 4$), Niddm1b ($n = 5$), and Niddm1i rats ($n = 5$) and incubated for 2 h with insulin (0–20,000 $\mu\text{U}/\text{ml}$). **A:** Glucose incorporation into lipids (lipogenesis) in the absence of insulin (basal conditions) was higher in F344 compared with GK ($P = 0.009$), Niddm1b ($P = 0.007$), and Niddm1i ($P = 0.04$); **B:** maximal insulin-induced lipogenesis was higher in F344 than in GK ($P = 0.00004$), Niddm1b ($P = 0.008$), and Niddm1i ($P = 0.001$). Maximal insulin-induced lipogenesis was higher in Niddm1b and Niddm1i compared with GK ($P = 0.02$ and 0.006 , respectively). Dose-dependent insulin-stimulated lipogenesis is expressed as increase above values obtained without insulin (**C**) (results are shown as mean \pm SE) and (**D**) as percent of maximum.

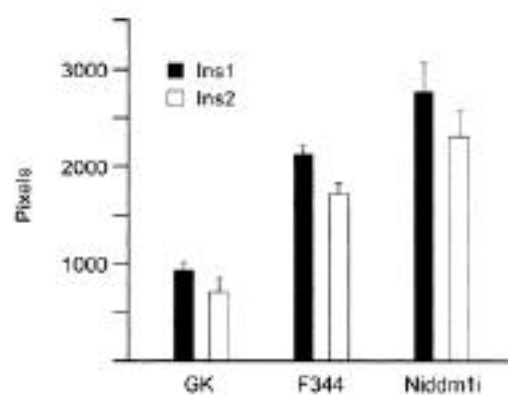


FIG. 4. Quantitative analysis of insulin RNA in congenic strains. Pancreatic RNA from four rats of each strain was isolated and the steady-state RNA levels were determined by RT-PCR using primers specific for the two rat insulin genes (*Ins1* and *Ins2*). Results are shown as mean \pm SE. The amounts of RNA are expressed as pixels calculated from band intensities using phosphoimaging technique.

or its associated phenotypes in the human or the mouse. Roughly guided by previously mapped genes on rat chromosome 1 and conserved synteny between rat, human, and mouse, we mapped three new genes to the *Niddm1* locus on rat chromosome 1 (indicated in bold in Fig. 1). These were the genes encoding Janus kinase 2 (JAK2) (chromosome 9p24 in human and 19 in mouse), 5-hydroxytryptamine receptor 7 (HTR7) (chromosome 10q24 in human and 19 in mouse), and pancreatic lipase (PNLIP) (chromosome 10q26.1 in human and 19 in mouse). This demonstrates homology between the *Niddm1* locus and human chromosome region 9p24 and, furthermore, confirms the syntenic conservation between rat chromosome 1, human chromosome region 10q24–26, and mouse chromosome 19.

DISCUSSION

In the present study, we have established congenic strains for the major GK diabetes locus, *Niddm1*, by introgression of GK alleles onto the genome of the normoglycemic F344 rat. We demonstrate that the major QTL (*Niddm1*) that explains 30% of the genetic effects on postprandial glucose concentration can be sorted into discrete genetic factors by establishing congenic strains covering different parts of the QTL. The data reveal a considerable complexity even within a single QTL in this well-defined model of type 2 diabetes. At least two genes with effects on the diabetic phenotype are located within the *Niddm1* locus, as demonstrated by the distinct phenotypes exhibited by the two nonoverlapping substrains Niddm1b and Niddm1i. Both congenic strains displayed elevated postprandial glucose levels and defects in insulin secretion in vivo as well as impaired basal and insulin-induced lipogenesis in isolated adipocytes. Given the difficulties in proving etiological relationships by pathophysiological studies, it is likely that the decreased insulin action measured in fat cells of 75-day-old rats reflects an early if not a primary event in the phenotype. Furthermore, several features are unique to the respective strain. Young Niddm1b and heterozygous Niddm1b/F344 rats have only slightly elevated postprandial glucose levels but substantially elevated insulin levels, indicating that insulin resistance is compensated by increased insulin secretion. In older heterozygous

rats the impaired insulin action can still be compensated but not in the homozygous *Niddm1b* rats, which develop fasting hyperglycemia, fasting hyperinsulinemia, increased body weight, and epididymal fat mass, as well as dyslipidemia. This constellation is well recognized in diabetes patients, in whom insulin resistance is considered as a cornerstone in the metabolic syndrome. The notion that insulin resistance in *Niddm1b* is a primary defect is supported by the fact that *Niddm1b/F344* heterozygous rats also exhibit signs of insulin resistance but display normal or below-normal levels of the other diabetes-associated phenotypes.

Insulin resistance and diabetes in humans are often associated with hypertriglyceridemia, increased levels of LDL cholesterol, and decreased levels of HDL cholesterol. The *Niddm1b* rats exhibit increased triglyceride levels in combination with increased total and HDL cholesterol. The difference between total and HDL cholesterol should approximately reflect the LDL and VLDL cholesterol levels, for which no difference was observed between *Niddm1b* and *F344* rats. Thus, the disordered lipid metabolism in *Niddm1b* does not exactly fit the pattern in diabetes patients. This discrepancy most probably reflects a species-specific difference in the manifestation of dyslipidemia in rodents as compared with humans.

The *Niddm1i* rats display insulin resistance in combination with a severe reduction of insulin secretion *in vivo*. This substrain of the *Niddm1* QTL did not develop increased body weight, epididymal fat mass, or increased levels of triglyceride. Thus, the phenotype is somewhat similar to that of patients with MODY with early defects in insulin secretion, although the mode of inheritance is apparently recessive since the insulin secretion defect was not observed in the *Niddm1i/F344* heterozygous rats. The insulin levels during IPGTT were already reduced in young *Niddm1i* rats, although the postprandial glucose levels were barely higher than in *F344*, possibly indicating an important contribution of insulin-independent glucose disposal at this age.

The generated *Niddm1* congenic strains are new and specific models for mild type 2 diabetes that will allow clarification of pathophysiological mechanisms of the disease. In diabetes patients as well as in GK rats, defects in both insulin secretion and action are implicated in the development of the disease (1,39–43). The relative etiological importance of these defects is still controversial. Since postprandial glucose levels are only slightly elevated and basal glucose is normal in 65-day-old *Niddm1i* rats, the defects in insulin secretion and action are most likely not merely consequences of glucotoxicity. Possibly, *Niddm1i* impairs mechanisms common to insulin secretion in pancreatic B-cells and insulin action in adipocytes. This would be analogous to the recent finding that inactivation of the gene encoding insulin receptor substrate 2 (*IRS-2*) causes defects in both insulin secretion and action in mice (44). The *IRS-2* gene is located on chromosome 13 in the human and chromosome 8 in the mouse (45,46). The corresponding homologous chromosomal regions to *Niddm1a* are 11q13, 9p24, and 10q24–26 in the human and chromosome 19 in the mouse. Thus, according to syntenic conservation, the *IRS-2* gene is not a candidate gene for the *Niddm1i* phenotype. Interestingly, a locus that was linked to diabetes in a Mexican-American population was recently reported on chromosome 10q (47). The authors also reported a locus with suggestive linkage to diabetes on

human chromosome 9p, which corresponds to *Niddm1b*. It is tantalizing to speculate that the same susceptibility genes may predispose to the diabetic phenotype in the GK rat and in human type 2 diabetes.

Furthermore, our data indicate the presence of nonallelic interaction or epistasis between the two diabetes loci *Niddm1b* and *Niddm1i*. In *Niddm1a* (encompassing both *Niddm1b* and *Niddm1i*), as compared to *F344*, the elevation of postprandial glucose levels was less severe than might be expected from the additive effect of the two substrains. Interpreting the epistasis in physiological terms suggests that counterregulatory mechanisms, which protect the organism against excessive glucose concentrations, restrict hyperglycemia unless the animals are carrying additional diabetes genes (as in GK) or are subjected to environmental stress. However, we cannot yet formally exclude that the GK-allele in the 2 cM region, unique to *Niddm1a* and defined by markers *Cyp2c12* and *D1Mgh29* (Fig. 1), has a glucose-lowering epistatic effect.

Despite the complex polygenic phenotype in the GK rat, we have demonstrated locus-specific phenotypes in the *Niddm1* collection of congenic strains. It is thus feasible to characterize pathophysiologically the contribution of this QTL to the complex disease phenotype, even before the identification of the actual disease-causing gene or genes. Prior knowledge of the phenotype profile of a QTL assists in the selection of candidate genes for subsequent studies. Furthermore, the retained phenotypes in our congenic strains assist in transforming the complex diabetic phenotype in GK into discrete bimodal traits that allow application of the same successful cloning strategies as for monogenic inherited diseases.

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