

# Deletion of Nicotinamide Nucleotide Transhydrogenase

## A New Quantitative Trait Locus Accounting for Glucose Intolerance in C57BL/6J Mice

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**The C57BL/6J mouse displays glucose intolerance and reduced insulin secretion. The genetic locus underlying this phenotype was mapped to nicotinamide nucleotide transhydrogenase (*Nnt*) on mouse chromosome 13, a nuclear-encoded mitochondrial protein involved in  $\beta$ -cell mitochondrial metabolism. C57BL/6J mice have a naturally occurring in-frame five-exon deletion in *Nnt* that removes exons 7–11. This results in a complete absence of *Nnt* protein in these mice. We show that transgenic expression of the entire *Nnt* gene in C57BL/6J mice rescues their impaired insulin secretion and glucose-intolerant phenotype. This study provides direct evidence that *Nnt* deficiency results in defective insulin secretion and inappropriate glucose homeostasis in male C57BL/6J mice. *Diabetes* 55:2153–2156, 2006**

C57BL/6J mice have been widely used as an inbred control strain in many studies. However, they also represent an important model of diet-induced diabetes, and they exhibit glucose intolerance, which is independent of obesity (1,2). Blood glucose levels of nonobese C57BL/6J mice, measured using an intraperitoneal glucose tolerance test (IPGTT), reveal that plasma glucose levels rise much higher and take longer to regain the resting level than in other mouse strains (3–5). The genetic defects that produce inappropriate homeostatic control in type 2 diabetes remain poorly understood, and, hence, animal models of glucose intolerance may provide valuable information about glucose homeostasis.

By intercrossing nonobese C57BL/6J and C3H/HeJ mice and using quantitative trait loci (QTLs) mapping, nicotinamide nucleotide transhydrogenase (*Nnt*) was identified

as a strong candidate gene (5). In C57BL/6J mice descended from the colony established at The Jackson Laboratory, we found that a spontaneous in-frame five-exon deletion in *Nnt*, also recently reported by Huang et al. (6), was linked to glucose intolerance and reduced insulin secretion. Although linkage analysis studies of complex traits cannot establish proof of QTLs identity at the molecular level, the genetic studies of defective *Nnt* in C57BL/6J mice strongly suggest a functional linkage between this gene and the observed phenotype (7). Here, we report transgenic rescue experiments demonstrating that defective *Nnt* is a QTL for glucose intolerance and impaired insulin secretion.

We rescued the *Nnt* deletion in C57BL/6J mice by transgenic expression of the entire *Nnt* gene sequence contained within a bacterial artificial chromosome (BAC). This BAC transgenic line was prepared using BAC RP22-455H18 derived from a 129S6/SvEvTac mouse BAC library obtained from Children's Hospital Oakland Research Institute. The BAC containing all 21 exons of the *Nnt* gene, as well as considerable 5' and 3' flanking intergenic DNA, was microinjected into the pronucleus of one-cell C57BL/6J embryos. Mice born were genotyped for the missing *Nnt* exons from tail-tip DNA, and offspring testing positive for the BAC were backcrossed to C57BL/6J. All mice were phenotyped at 12 and 16 weeks of age by IPGTT for plasma glucose and insulin responses.

### RESEARCH DESIGN AND METHODS

**Animal husbandry.** Mice were kept in accordance with U.K. Home Office welfare guidelines and project license restrictions under controlled light (12-h light and 12-h dark cycle), temperature ( $21 \pm 2^\circ\text{C}$ ), and humidity ( $55 \pm 10\%$ ) conditions. They had free access to water (25 ppm chlorine) and were fed ad libitum on a commercial diet (SDS maintenance diet) containing 2.6% saturated fat.

**Production of BAC transgenic mice.** Transgenic mice were generated by pronuclear injection of closed circular BAC DNA (available at <http://bacpac.chori.org/home.htm>) into mouse C57BL/6J one-cell embryos. Briefly, 3-week-old C57BL/6J mice were superovulated with 5 units pregnant mare's serum gonadotropin, followed 46 h later by 5 units human chorionic gonadotropin and mated with C57BL/6J males overnight. The next day, females with copulation plugs were culled and embryos harvested and treated with hyaluronidase to remove cumulus cells (8). DNA prepared using a Qiagen Large Construct kit was resuspended in polyamine buffer (10 mmol/l Tris-HCl pH 7.5, 0.1 mmol/l EDTA, pH 8.0, 100 mmol/l NaCl, 30  $\mu\text{mol/l}$  spermine, and 70  $\mu\text{mol/l}$  spermidine) immediately before microinjection. Between 100 and 200 embryos were injected with BAC DNA at a concentration of 1.0 ng/ $\mu\text{l}$ . Injected embryos were transferred on the same day to the oviducts of pseudopregnant CD1-recipient females. Litters arising from these transfers were genotyped at 3 weeks of age using ear biopsies to identify transgenic animals.

**DNA extraction and genotyping.** Genomic DNA was extracted from mouse tail tissue using a Qiagen DNeasy tissue kit. Mice were genotyped by PCR of

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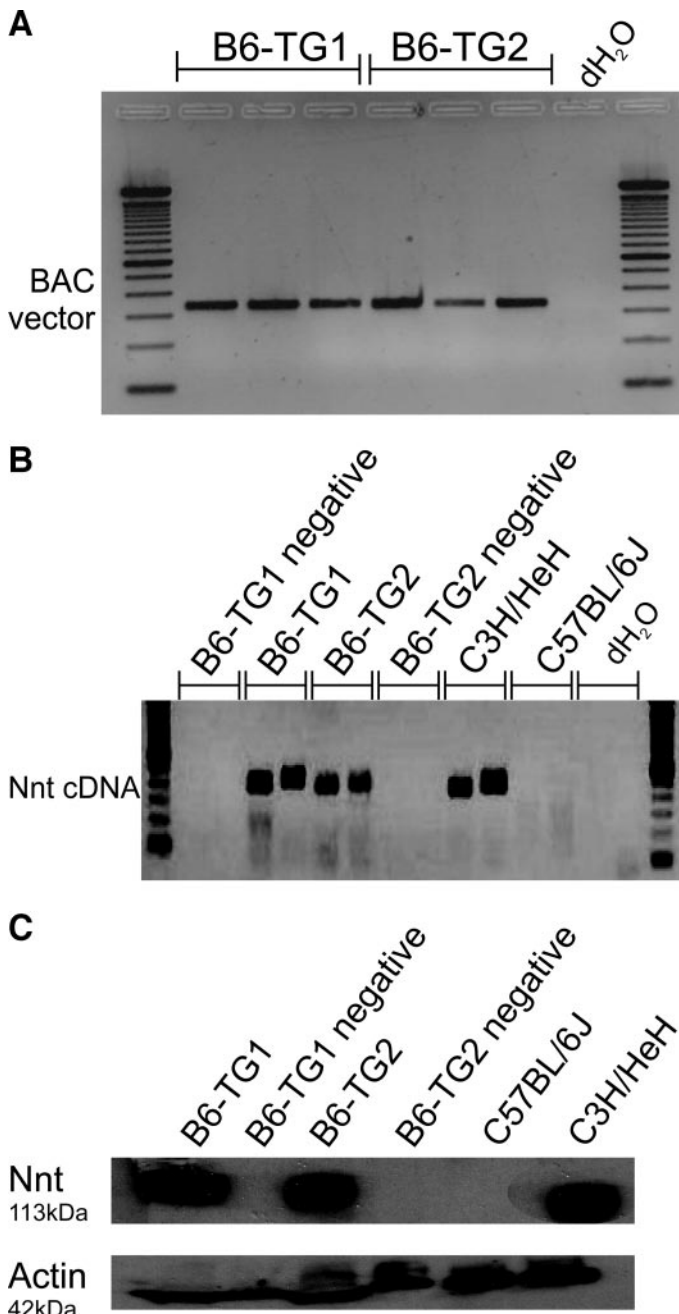
Additional information for this article can be found in an online appendix at <http://diabetes.diabetes.org>.

BAC, bacterial artificial chromosome; IPGTT, intraperitoneal glucose tolerance test; *Nnt*, nicotinamide nucleotide transhydrogenase; QTLs, quantitative trait loci.

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**FIG. 1.** BAC transgenic expression of wild-type *Nnt* in male C57BL/6J mice. **A:** PCR of BAC vector pBACe3.6 (U80929) using three primer sets of equal product size targeted to the start, middle, and end of the vector sequence. All three bands are clearly present in the C57BL/6J mice carrying the entire *Nnt* gene sequence (B6-TG1 and B6-TG2), whereas in the C57BL/6J littermates without the transgene (B6-TG-negative controls) there was no amplification (online appendix c). This confirms that the presence or absence of missing *Nnt* exons is due solely to the presence of the BAC. **B:** RT-PCR analysis of *Nnt* cDNA expression across missing exons 7–11 endogenous to the C57BL/6J mouse. The naturally occurring multiexon deletion in the C57BL/6J strain is too large, even at the cDNA level, to be amplified easily in a single PCR, so it was amplified as two fragments both spanning several intronic regions. Hence, two bands are visible for the C57BL/6J expressing the BAC (B6-TG1 and B6-TG2), and both bands are absent in their littermate controls (B6-TG1 negative and B6-TG2 negative). C3H/HeH was used as a positive control, as C3H/HeH mice express all 21 exons of the *Nnt* gene. Wild-type C57BL/6J and deionized water were run as negative controls. **C:** Western blot showing abundant expression of wild-type *Nnt* protein in liver tissue from B6-TG1 and B6-TG2 mice, using C3H/HeH mice as a positive control. No expression was detectable in livers of B6-TG1-negative and B6-TG2-negative strains nor in livers of noninjected C57BL/6J mice used as a negative controls.

missing exons 7 and 11 of the *Nnt* gene. Primers listed in 5' to 3' orientation are for *Nnt* exon 7: NntXn7 forward gtcattgaacctcaaaag and NntXn7 reverse caggaagaagctctgtttt; for *Nnt* exon 11: NntXn11 forward tctctgattctctctct and NntXn11 reverse gctgccttgacttggatt. PCR products were visualized on a 2% agarose gel run at 200V for 25 min.

**Reverse transcriptase-PCR.** Total RNA from mouse liver was extracted using an RNeasy mini-kit (Qiagen). cDNA generated by Superscript II enzyme (Invitrogen) underwent PCR amplification across the deleted fragment of *Nnt* and several introns. Primers listed in 5' to 3' orientation are for *Nnt* fragment 1 forward cagcacagctctgattccag and *Nnt* fragment 1 reverse gtaaacccaagac cgtggctgag; for *Nnt* fragment 2 forward gtgatgaaggatggcaaatg and *Nnt* fragment 2 reverse gcaggtggtttctggtgactc.

**IPGTTs.** Mice were fasted overnight, weighed, and a blood sample collected by tail venipuncture under local anesthetic (Lignocaine) using lithium-heparin microvette tubes (Sarstedt) to establish a baseline glucose level "T0." They were then injected intraperitoneally with 2 g glucose (20% glucose in 0.9% NaCl) per kilogram body weight and blood samples taken at the designated times. For a fuller protocol, see EMPReSS (the European Mouse Phenotyping Resource for Standardized Screens designed by Eumorphia)-simplified IPGTT (available at <http://empress.har.mrc.ac.uk>). Plasma glucose was measured using an Analox Glucose analyzer GM9 (Analox, London, U.K.). Plasma insulin was measured using a Mercodia Ultra-Sensitive Mouse ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions.

**Islet isolation.** Mice were killed by cervical dislocation, the pancreas removed, and islets isolated by liberase digestion and handpicking (5). Isolated islets were dispersed into single cells by incubation in calcium-free Hank's solution (1 mmol/l EGTA) and trituration in RPMI tissue culture medium containing 5.5 mmol/l glucose (Life Technologies, Paisley, U.K.) supplemented with 10% FCS, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were maintained in this medium at 37°C in a humidified atmosphere at 5% CO<sub>2</sub> in air and used 1–2 days after the isolation.

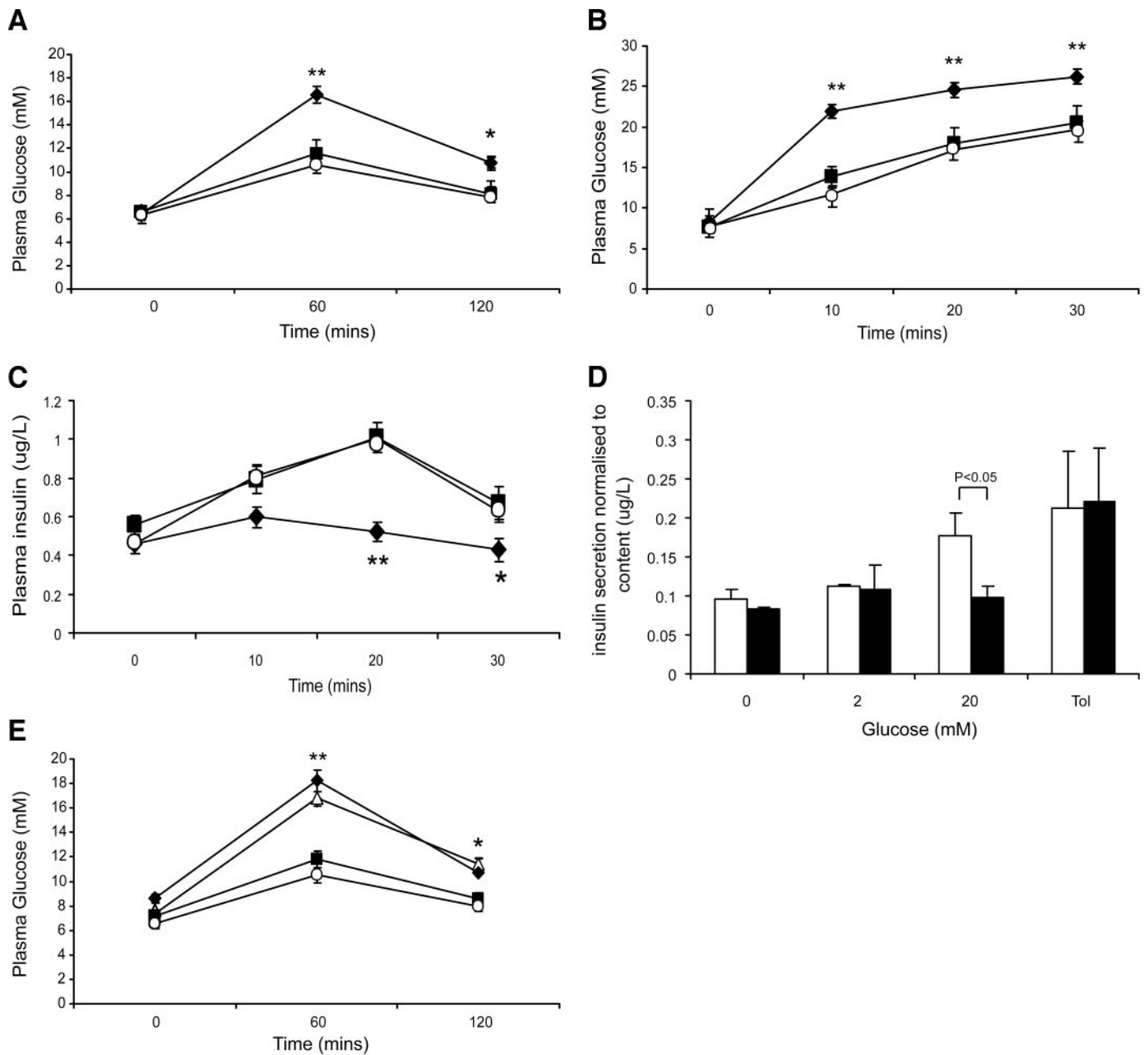
**Protein gels and Western blot analysis.** *Nnt* expression was verified using SDS-PAGE 12% gel and Western blot. Protein samples were heated for 3 min at 100°C in sample buffer (final concentration of 30 mmol/l Tris-HCl, pH6.8, 5% glycerol, 0.005% bromophenol blue, and 2.5%  $\beta$ -mercaptoethanol). Proteins were size separated by electrophoresis alongside a "SeeBlue" (Invitrogen, Paisley, U.K.) protein ladder to allow molecular weights to be estimated.

Proteins were transferred to hybond-P membrane (Amersham, Chalfont St. Giles, U.K.) using a Transblot SD system (Biorad) by passing 100 V across membrane/gel for 1 h. Membranes were probed with a custom-made polyclonal *Nnt* antibody raised against amino acids 10731-086 at the C-terminus of the peptide (Eurogentec, Seraing, Belgium) and actin antibody (control) from Santa Cruz (Santa Cruz, CA). Enhanced Chemiluminescence Plus (Amersham) was then used according to manufacturers' instructions to allow visualization of protein on enhanced chemiluminescence film, developed using the Compact X4 (Xograph).

## RESULTS

We obtained two independent transgenic lines, B6-TG1 and B6-TG2. BAC complementation was verified first by PCR of the BAC vector itself (Fig. 1A) and second at the transcriptional level by detection of cDNA missing exons 7–11 in the *Nnt* gene (Fig. 1B). This was also observed at the protein level by Western blotting. The amount of *Nnt* protein produced by both transgenic lines was similar to or higher than that produced by a C3H/HeH control strain that harbors wild-type *Nnt* (Fig. 1C). *Nnt* protein was completely absent in B6-TG-negative controls and stock C57BL/6J mice, as previously reported (6). Both the transgenes were transmitted through the germline, and lines were maintained by crossing to wild-type C57BL/6J mice.

In both lines, expression of wild-type *Nnt* on the C57BL/6J background in male mice induced improvements in glucose tolerance following a glucose challenge at 12 and 16 weeks of age compared with nontransgenic littermates (Fig. 2A and B). We therefore looked for differences in insulin secretion by carrying out an IPGTT taking samples at 0, 10, 20, and 30 min after glucose injection (Fig. 2C). Insulin secretion by B6-TG1 and B6-TG2 mice was significantly higher (for the same glucose challenge per gram bodyweight) than for B6-TG-negative mice. No difference in body weight was observed between the



**FIG. 2.** Plasma glucose and insulin dynamics in wild-type and C57BL/6J male BAC transgenic mice. **A:** IPGTT curves obtained in two transgenic lines expressing wild-type *Nnt* on the genetic background of the C57BL/6J strain that harbors the deletion variant of *Nnt* (■, B6-TG1 [ $n = 12$ ]; ○, B6-TG2 [ $n = 10$ ]) and in C57BL/6J littermates without the transgene (◆, B6-TG-negative controls [ $n = 32$ ]). Data points indicate the mean  $\pm$  SE. Statistical significance of B6-TG-negative compared with the other groups is indicated; \* $P < 0.05$  and \*\* $P < 0.01$  (two-tailed Student's *t* test). **B and C:** Plasma glucose (**B**) and insulin (**C**) dynamics measured in response to an IPGTT at 16 weeks for the same mice as in Fig. 1A. The IPGTT was carried out over 30 min. For key, see A. Data points indicate the means  $\pm$  SE. **D:** Insulin secretion from isolated islets in response to glucose (0, 2, 20 mmol/l) or tolbutamide (200  $\mu$ mol/l). Islets were isolated, as previously described (7). Each data point is the mean of six measurements (each using five islets) and is representative of two separate experiments on B6-TG1 mice and two on B6-TG2 mice. □, B6-TG1 mice; ■, B6-TG-negative control littermates. Data points indicate the means  $\pm$  SE. Statistical significance at 20 mmol/l ( $P = 0.034$ ) determined by two-tailed Student's *t* test. **E:** Plasma glucose dynamics measured in response to an IPGTT at 12 weeks of age (as for A) comparing the *Nnt* BAC transgenic mice with male C3H/HeH and C57BL/6J mice. The IPGTT was carried out over 120 min. ○, B6-TG2 ( $n = 10$ ); ◆, B6-TG-negative control littermates ( $n = 32$ ); △, C57BL/6J mice ( $n = 18$ ); ■, C3H/HeH mice ( $n = 10$ ). Data points indicate the means  $\pm$  SE. Statistical significance of B6-TG-negative mice compared with B6-TG2 mice is indicated; \* $P < 0.05$  and \*\* $P < 0.01$  (two-tailed Student's *t* test).

transgenic lines compared with the C57BL/6J littermates without the transgene. Similar results were seen in female C57BL/6J mice, although glucose intolerance in females is less pronounced (5) (online appendix *d* [available at <http://diabetes.diabetesjournals.org>]).

To confirm that a defect in  $\beta$ -cell function, due to a deletion in *Nnt*, underlies the glucose intolerance of

C57BL/6J mice and that this is ameliorated in our transgenic rescue mice, we compared the ability of glucose to stimulate insulin secretion from islets isolated from B6-TG and B6-TG-negative mice. Figure 2D shows that there was no difference in basal insulin secretion (at 0 and 2 mmol/l glucose) between the two mouse strains but that insulin secretion in response to 20 mmol/l glucose was increased

in B6-TG islets in comparison to B6-TG-negative islets ( $P < 0.05$ ). The ATP-sensitive  $K^+$  channel blocker tolbutamide stimulated secretion comparably in both strains as expected because it bypasses the steps in metabolism secretion coupling that involve *Nnt* (7,9,10). Figure 2E clearly shows that B6-TG mice have normal glucose tolerance resembling that of C3H/HeH control mice, whereas both B6-TG-negative and C57BL/6J mice display significantly higher plasma glucose levels after 60 and 120 min following IPGTT. Of 23 mouse strains previously screened (5), including six other C57BL mouse strains, only C57BL/6J had the naturally occurring deletion in *Nnt* (online appendix a).

## DISCUSSION

Our findings show that the deletion of *Nnt* observed in C57BL/6J mice accounts for their glucose insensitivity and reduced insulin secretion. This supports the concept that primary genetic defects in  $\beta$ -cell metabolism contribute to the pathogenesis of impaired glucose homeostasis (11,12). Our QTL mapping and that of Kayo et al. (13) suggested that this locus would account for  $\sim 10\%$  of the variance of the phenotype (5,13). However, this is in the  $F_2$  genetic environment rather than a pure C57BL/6J genetic environment, where *Nnt* alone appears to be sufficient to account for the phenotype. Thus, the genetic compositions of the mice are different, and this illustrates the importance of genetic interactions in determining quantitative traits.

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