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Susceptibility to streptozotocin-induced diabetes is mapped to mouse chromosome 11

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Abstract

To study the contribution of β-cell vulnerability to susceptibility to diabetes, we studied β-cell vulnerability to a single high dose of streptozotocin (STZ) in an animal model of type 2 diabetes, the NSY mouse, a sister strain of the STZ-sensitive NOD mouse, in comparison with the STZ-resistant C3H mouse. NSY mice were found to be extremely sensitive to STZ. Introgression of a single Chr 11, where STZ-sensitivity was mapped in the NOD mouse, from NSY mice converted STZ-resistant C3H mice to STZ-sensitive. Two nucleotide substitutions were identified in the nucleoredoxin gene, a positional and functional candidate gene for STZ-induced diabetes on Chr 11. These data, together with the co-localization of type 1 (*Idd4*) and type 2 (*Nidd1n*) susceptibility genes on Chr 11, suggest that the intrinsic vulnerability of pancreatic β cells is determined by a gene or genes on Chr 11, which may also contribute to susceptibility to spontaneous diabetes.

Keywords: β-cell vulnerability; Congenic strain; Consomic strain; *Idd4*; *Nidd1n*; Nucleoredoxin; STZ susceptibility

Streptozotocin (STZ) has been widely used to induce diabetes in experimental animals. Among inbred strains of mice, varying susceptibility to STZ-induced diabetes has been reported, indicating the importance of genetic background in β-cell destruction by STZ. The nonobese diabetic (NOD) and C57BL/6 strains were reported to be extremely susceptible to β-cell destruction by STZ, while the C3H mouse is particularly resistant to this drug [1,2]. Since several lines of evidence suggested that β-cell destruction by STZ is mediated by locally expressed reactive oxygen intermediates (ROIs) [3], a difference in the ability to protect β-cells from oxidative stress is among the possible explanations for strain difference in susceptibility to STZ-induced diabetes.

The NOD mouse spontaneously develops autoimmune type 1 diabetes with remarkable similarities to human type 1 diabetes [4,5]. In both the NOD mouse and humans, the development of diabetes is under polygenic control, with environmental factors influencing penetrance [6]. To date, more than 20 susceptibility loci for type 1 diabetes have been mapped in the NOD mouse [6–11]. Although susceptibility to type 1 diabetes is primarily determined by dysregulation of the immune system, several studies suggested that pancreatic β-cells themselves may also be involved in determining susceptibility to type 1 diabetes through the expression of vulnerability genes, such as genes related to oxidative stress [12–14]. Such genes may be involved in the

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intrinsic vulnerability of pancreatic β-cells to the immune destructive process in type 1 diabetes, as well as to destruction by β-cell toxins such as STZ. In fact, we have previously reported that targeted expression of an anti-oxidative molecule, thioredoxin, in pancreatic β-cells is protective against autoimmune type 1 diabetes as well as STZ-induced diabetes [15]. Moreover, Gonzalez et al. [2,16] recently reported that a susceptibility locus for STZ-sensitivity on chromosome (Chr) 11 co-localized with a susceptibility locus for type 1 diabetes, *Idd4*, mapped in the NOD mouse (Fig. 1), suggesting that *Idd4* could be involved in a common vulnerability pathway leading to STZ- and autoimmune-mediated pancreatic β-cell destruction.

Recent studies have implicated a role of oxidative stress in not only β-cell destruction in autoimmune type 1 diabetes, but also β-cell failure due to chronic hyperglycemia (glucose toxicity) in type 2 diabetes [17–19]. The Nagoya–Shibata–Yasuda (NSY) mouse, an inbred animal model of type 2 diabetes, was established by selective breeding for glucose intolerance from an outbred colony, Jcl:ICR mice, from which an animal model of type 1 diabetes, the NOD mouse [4], was also derived [20,21]. The genetic control of type 2 diabetes in the NSY mouse is also polygenic, and one of the susceptibility genes, *Nidd1n*, has been mapped to Chr 11 [22,23]. The support interval for *Nidd1n* overlaps the region where a susceptibility gene for type 1 diabetes, *Idd4*, and one for STZ-induced diabetes were mapped in the NOD mouse [2,11,23] (Fig. 1). Although type 1 and type 2 diabetes are considered clinically and etiologically distinct diseases, several lines of evidence have suggested common genetic factors between the two types of diabetes in humans [24,25]. Although each type of diabetes certainly has a few major susceptibility genes specific to each type, some other genes with a modest effect or modifiers may have segregated from a common ancestor to both strains, contributing to type 1 diabetes in the NOD mouse and type 2 diabetes in the NSY mouse as a common genetic factor. The co-localization of a type 1 diabetes gene, *Idd4*, in the NOD mouse and a type 2 diabetes gene, *Nidd1n*, in the NSY mouse in the same region on Chr 11 therefore suggests the possibility that *Idd4* and *Nidd1n* convey shared susceptibility to both type 1 and type 2 diabetes. Given the common mechanisms of β-cell failure in type 1 and type 2 diabetes as well as in STZ-induced diabetes, defensive mechanisms against oxidative stress are among possible mechanisms for the shared susceptibility to type 1, type 2, and STZ-induced diabetes.

In this study, we investigated the contribution of β-cell vulnerability to susceptibility to diabetes in three ways. First, we studied β-cell vulnerability to STZ in the NSY mouse, an inbred animal model of type 2 diabetes derived from the same outbred colony as the NOD mouse, and therefore potentially sharing the same susceptibility to STZ-induced diabetes as the NOD mouse. Second, after confirming susceptibility to STZ-induced diabetes in the NSY mouse, we studied whether or not a single chromosomal substitution converts STZ-resistance of the C3H mouse to STZ-sensitivity in a consomic strain, C3H-11^{NSY}, which carries an NSY-derived Chr 11 on a C3H-derived STZ-resistance background. Third, since the nucleoredoxin gene (*Nxn*), mapped at 45.2-cM on mouse Chr 11 [26] (Fig. 1), is a positional and functional candidate gene for STZ-induced diabetes, we determined the nucleotide sequences of the entire coding region as well as 5′-upstream and 3′-downstream regions in NOD, NSY, and C3H mice. In addition to being a candidate for STZ-induced diabetes, the possibility of *Nxn* conferring common susceptibility between type 1 (*Idd4*) and type 2 (*Nidd1n*) diabetes was also tested by comparing sequences among these strains.

Materials and methods

Animals. NSY mice were originally obtained from the Branch Hospital of Nagoya University School of Medicine. C3H/He (C3H) mice were purchased from Charles River Laboratories (Kanagawa, Japan). NOD mice were obtained from Shionogi AC Center (Shiga, Japan). These mice were maintained in the animal facilities of Osaka University Graduate School of Medicine, and Osaka University Graduate School Guidelines for the Care and Use of Laboratory Animals were followed. All mice had free access to tap water and a

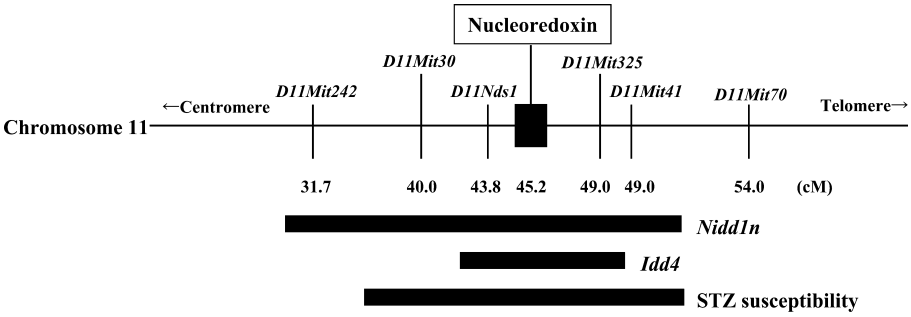

Fig. 1. Support interval for *Idd4* [11], *Nidd1n* [23], and STZ-sensitivity [2] and position of *Nxn* [26]. Map distances of *Nxn* and microsatellite markers from the centromere (in cM) are derived from the Mouse Genome Database (http://www.informatics.jax.org).

standard diet (CRF-1: Oriental Yeast, Tokyo, Japan) in an air-conditioned room (22–25 °C) with a 12-h light–dark cycle (6:00–18:00 h). Male mice were used for all experiments.

Development of consomic strain. A consomic strain C3H-11^{NSY} was produced by mating (NSY × C3H)F1 with C3H, and selecting males that were heterozygous for Chr 11. These males were then mated with C3H females, and their male progeny, heterozygous for Chr 11, were used for the next generation. This process was repeated until generation N6 or N7, at which point mice heterozygous for Chr 11 were intercrossed to obtain homozygotes for Chr 11. This line has been maintained by brother–sister mating.

Genotype analysis and localization of markers. Genomic DNA was extracted from the tail with standard phenol chloroform methods. Information on microsatellite (simple sequence length polymorphisms) markers was obtained from the Mouse Genome Database (http://www.informatics.jax.org). The markers were amplified by polymerase chain reaction (PCR) with primers purchased from Research Genetics (Huntsville, AL). A total of 74 informative marker loci spanning the whole mouse genome were analyzed. For Chr 11, 16 markers were used to confirm no recombination (average span less than 5 cM). PCR was performed essentially based on the manufacturer's protocol, and the PCR products were electrophoresed on 9% polyacrylamide gels and visualized by ethidium bromide staining.

STZ injection and blood glucose measurement. NSY, C3H, and C3H-11^{NSY} mice received a single injection of STZ at a dose of 175 mg/kg body weight at 12 weeks of age. Male NSY mice were used because they were more susceptible to type 2 diabetes and male NOD mice were more sensitive to STZ. STZ was dissolved in sodium citrate buffer (Wako Pure Chemical Industries, Osaka, Japan) and immediately injected intraperitoneally. Blood glucose level and body weight were measured on days 0, 1, 2, 4, 5, 7, and 8 after injection. Mice with a blood glucose level higher than 16.7 mmol/L were considered diabetic.

DNA isolation and synthesis of first strand cDNA. Genomic DNA was extracted from the liver of NOD, NSY, and C3H/He mice by standard methods. Total RNA was isolated from the kidney using TRIZOL (Gibco-BRL, Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol. First strand cDNA was synthesized using total RNA, random primers, dNTPs, and ReverTra Ace (TOYOBO, Osaka, Japan) at 30 °C for 10 min, 42 °C for 20 min, 99 °C for 5 min, and 4 °C for 5 min.

Sequence analysis. Five pairs of primers were designed from the published sequence of *Nxn* in the C57BL/6 strain [26] (Table 1), so that the 5′-upstream region and coding region of *Nxn* were covered by five overlapping segments. PCR amplification was carried out using 100 ng genomic DNA for segment 1, 2 μl synthesized cDNA for segment 2, 60 ng upstream and downstream primers, buffer, dNTPs, and 2.5 U LA *Taq* polymerase (TAKARA SHUZO, Shiga, Japan). Thirty cycles of amplification were performed, each consisting of 30 s at 96 °C, 30 s at 55 °C, and 30 s at 72 °C. PCR amplification of segments 3, 4, and 5 was performed using 2 μl synthesized cDNA, 60 ng upstream and downstream primers, buffer, dNTPs, and 2.5 U KOD Dash *Taq* polymerase (TOYOBO). Thirty cycles of amplification were performed, each consisting of 30 s at 94 °C, 2 s at 55 °C, and 30 s at 74 °C. Amplified PCR products were purified using a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). Sequencing reaction was performed using Big Dye Terminator (PE, Foster City, CA, USA) according to the manufacturer's protocol, and the products were directly sequenced using an ABI 3100 sequencer (PE, Foster City, CA, USA).

Statistical analysis. Statistical analysis was performed by Mann–Whitney *U* test. Life table analysis was performed to compare the cumulative incidence of diabetes.

Results

STZ sensitivity in NSY and C3H mice

NSY mice showed a significantly larger increase in blood glucose level from baseline at all time points after STZ injection than C3H mice (Fig. 2). Following the

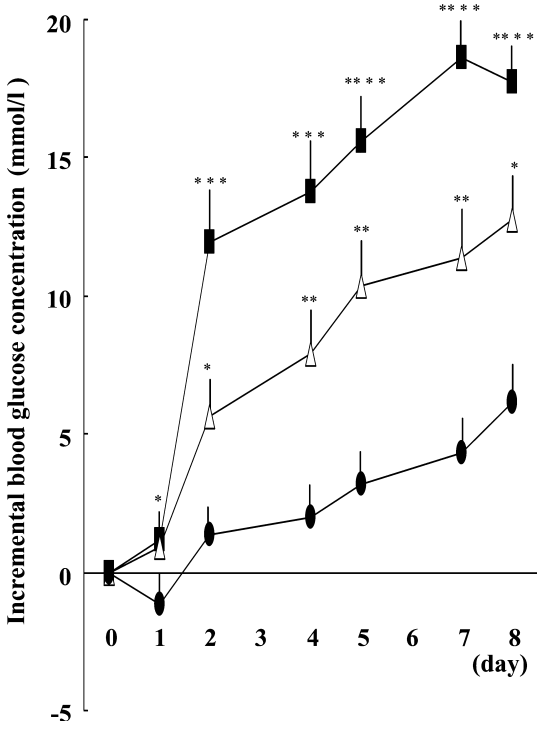

Fig. 2. Analysis of incremental blood glucose concentration after STZ-injection in NSY ($n = 17$; black squares), C3H-11^{NSY} ($n = 23$; white triangles), and C3H ($n = 20$; black circles) mice. Glucose concentration was determined 0, 1, 2, 4, 5, 7, and 8 days after a single intraperitoneal injection of STZ at 175 mg/kg body weight at 12 weeks of age. Values are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs. C3H mice, Mann–Whitney *U* test. The significant difference on day 1 is for NSY vs. C3H mice.

Table 1
Sequences of polymerase chain reaction primers used for amplification of *Nxn*

Region	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
Segment 1	ATGGAGGGGAGTGTCTGG	CTGCACCCGAAGTAGAGC	273
Segment 2	GGAGGTGGACGTGCATTC	ACGAAGTCCTGCCACTGC	255
Segment 3	CGCCTGGAGATCGTCTTC	TGGCCTGCCTCCTTGAT	431
Segment 4	TGCTGGTGGAATCCTACC	TGGCTGCCTCGGACTCTC	393
Segment 5	CTGACTCCAACGCTGTGC	CTGCCACTGTGAGGATGC	542

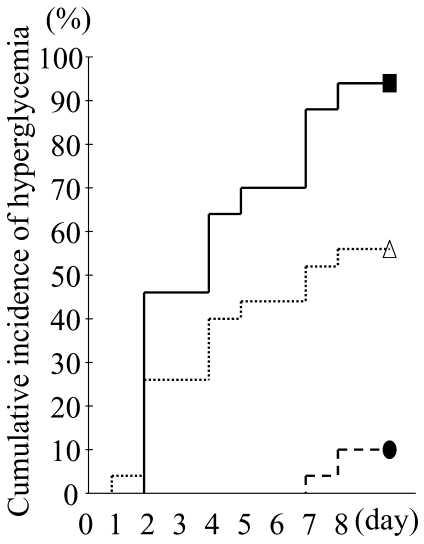

Fig. 3. Cumulative incidence of hyperglycemia after single intraperitoneal injection of STZ (175 mg/kg body weight) in NSY ($n = 17$; black squares), C3H-11^{NSY} ($n = 23$; white triangles), and C3H ($n = 20$; black circles) mice. Glucose concentration was determined 0, 1, 2, 4, 5, 7, and 8 days after a single intraperitoneal injection of STZ at 12 weeks of age. Animals were considered hyperglycemic when blood glucose level was >16.7 mmol/L. Data are presented as the percentage of hyperglycemic mice among all treated mice at the indicated time points. At 8 days after STZ-injection, diabetes incidence was 94% (16 of 17) in NSY mice, 57% (13 of 23) in C3H-11^{NSY} mice, and 10% (2 of 20) in C3H mice.

administration of STZ, about half of NSY mice (8/17) had become diabetic by day 2 whereas none of C3H mice had developed diabetes, and almost all NSY mice (>90%) had developed diabetes by 8 days after STZ injection, whereas only two C3H mice had developed diabetes (2/20) (Fig. 3, $p < 0.0001$). NSY mice showed a significant decrease in body weight (39.4 g vs. 35.8 g, $p < 0.005$) due to marked glycosuria at 8 days after STZ injection, whereas no significant change was observed in C3H mice (24.0 g vs. 24.4 g, $p = 0.21$).

STZ sensitivity in consomic strain

C3H-11^{NSY} mice showed a significantly larger increase in blood glucose level from baseline at all time points after STZ injection than C3H mice (Fig. 2), indicating that introgression of a single chromosome (Chr 11) from STZ-sensitive NSY mice converted STZ-resistant C3H mice to STZ-sensitive. The cumulative incidence of STZ-induced diabetes was significantly higher in C3H-11^{NSY} mice than in C3H mice (Fig. 3, $p < 0.005$). Compared with NSY mice, however, C3H-11^{NSY} mice showed significantly lower blood glucose levels on days 7 and 8 after STZ injection, and life table analysis demonstrated a significant difference between the survival curves (Fig. 3, $p < 0.05$), indicating that sensitivity of C3H-11^{NSY} mice to STZ is not as strong as that in NSY mice, suggesting that chromosomes other than Chr 11 may also contribute to STZ sensitivity.

Table 2
Summary of sequence variants in nucleoredoxin gene of five inbred strains

	Exon	3′-UTR
Position:	456	1549
C57BL/6, NOD	C	C
NSY, C3H/He	A	T

Blood glucose levels of heterozygous C3H-11^{NSY} mice were intermediate between those of homozygous C3H-11^{NSY} and C3H mice, suggesting that the mode of inheritance of STZ-sensitivity is additive.

Sequence of nucleoredoxin

The nucleotide sequences determined were 1603 bp in length in NOD (Accession Nos. AB095441 and AB096016), NSY (Accession Nos. AB095442 and AB096017), and C3H/He (Accession Nos. AB095444 and AB096019) mice, which spanned the 5′ upstream region, the coding region, and a part of the 3′-untranslated region (UTR) of *Nxn*. These segments were compared with the reported sequence in the C57BL/6 mouse (http://www.ensembl.org/Mus_musculus). Two nucleotide substitutions, one in the coding region and one in 3′-UTR, were identified, giving two different haplotypes in the four strains studied (Table 2). The nucleotide sequence in the NSY mouse, an animal model of type 2 diabetes, was different from the reported sequence in the C57BL/6 mouse: with a C to A change in the coding region at position 456 and a C to T change in 3′-UTR at position 1549 (numbering is according to that in EMBL, Accession No. X92750) [26]. The substitution in the coding region did not lead to an amino acid change. The same substitutions were detected in the C3H/He mouse, but not in the NOD mouse.

Discussion

In this study, the NSY mouse, an animal model of type 2 diabetes, derived from the same outbred colony as the NOD mouse [20–22], was shown to be extremely susceptible to diabetes induced by a single injection of a high dose of STZ. NOD mice are also reported to be highly sensitive to STZ, indicating that the two strains, NOD and NSY, derived from the same outbred colony share the same characteristics of intrinsic vulnerability of β-cells to STZ and susceptibility to STZ-induced diabetes. The conversion of STZ-resistant C3H mice to STZ-sensitive by the introgression of a single chromosome, Chr 11, from STZ-sensitive NSY mice indicates that STZ-sensitivity is determined, at least in part, by a gene or genes on Chr 11 of the NSY mouse. Recent

studies by Gonzalez et al. have shown that STZ-sensitivity in the NOD mouse is also mapped to Chr 11. The co-localization of susceptibility genes for STZ-diabetes on Chr 11 in NOD and NSY mice derived from the same closed colony suggests the possibility that the genes predisposing to STZ-induced diabetes may have segregated from a common ancestor, contributing to susceptibility to STZ-induced diabetes in both strains.

The present study with consomic mice, in which Chr 11 of the STZ-sensitive NSY mouse was introgressed onto the STZ-resistant C3H genetic background, clearly demonstrated that Chr 11 harbors susceptibility to STZ-induced diabetes in the NSY mouse. STZ-sensitivity in the consomic mice, however, was not as strong as that in the NSY parental strain, suggesting that STZ-induced diabetes is under polygenic control, and that other genes on different chromosomes in addition to Chr 11 may also contribute to STZ-sensitivity. STZ-sensitivity in the NOD mouse was also reported to be under polygenic control, with a contribution from Chr 9 in addition to Chr 11. Thus, in both the NOD and NSY mice, susceptibility to STZ-induced diabetes is under polygenic control, one gene of which is mapped to Chr 11.

Several lines of evidence have suggested that locally produced ROIs are involved in the mechanisms of pancreatic β-cell destruction in STZ-induced diabetes [3]. Genes involved in oxidative stress and free radical scavengers are therefore strong candidate genes for STZ-induced diabetes. The nucleoredoxin gene (*Nxn*), mapped at 45.2cM on mouse Chr 11 [26] (Fig. 1), is therefore a positional and functional candidate gene for STZ-sensitivity, because it encodes a nuclear protein with structural and biochemical similarities to thioredoxin [26–28], which controls redox regulation and is induced by various types of stresses, such as viral infection, hypoxia, and ROIs [29–33]. Moreover, we have previously reported the prevention of STZ-induced diabetes by targeted overexpression of thioredoxin in pancreatic β-cells [15], indicating a role of thioredoxin in the development of STZ-induced diabetes through redox regulation. Given its structural and biochemical similarity to thioredoxin, nucleoredoxin may also be involved in susceptibility to STZ-induced diabetes. To examine whether *Nxn* is responsible for susceptibility to STZ-induced diabetes, we analyzed the sequence of *Nxn* in NOD and C3H mice, which were used to map the STZ-sensitivity gene on Chr 11 [2], and detected two different variants. Although the C to A change in the coding region does not lead to an amino acid change, the C to T change in 3′-UTR may have a functional effect through a difference in transcriptional activity. Further studies on the functional significance of this variant, such as reporter assay, are needed to confirm this possibility.

The co-localization of susceptibility loci for STZ-induced diabetes in the NOD mouse [2] and NSY mouse (this study), as well as those for type 1 diabetes in the NOD mouse (*Idd4*) [11] and for type 2 diabetes in the NSY mouse (*Nidd1n*) [23], in the same region on chromosome 11 suggests the possibility that these genes may convey shared susceptibility to type 1, type 2, and STZ-induced diabetes. Functional data for *Idd4* and *Nidd1n* also suggest this possibility because *Idd4* is linked to diabetes but not insulitis [34], suggesting that *Idd4* contributes to vulnerability of β-cells under autoimmune attack, but not to the initiation of autoimmunity. *Nidd1n* is reported to be linked to β-cell dysfunction but not insulin resistance [23], suggesting that *Nidd1n* also contributes to β-cell vulnerability to glucose toxicity and/or insulin resistance. In addition to STZ-induced diabetes, recent studies have implicated a role of oxidative stress in β-cell dysfunction in type 2 diabetes as well as autoimmune type 1 diabetes [15,35–37]. We have previously reported that targeted overexpression of an antioxidative molecule, thioredoxin, prevented type 1 diabetes in the NOD mouse [15]. Ihara et al. [17] reported that oxidative stress due to chronic hyperglycemia damaged pancreatic β-cells in a rat model of type 2 diabetes, and that antioxidant treatment had beneficial effects on glycemic control [18]. Kaneto et al. [19] reported that antioxidant treatment prevented β-cell dysfunction in a mouse model of type 2 diabetes. These data suggest the possibility that mutations in antioxidative molecules, such as nucleoredoxin, may be involved in the pathogenesis of not only STZ-induced diabetes but also type 1 and type 2 diabetes. It is possible that mutations in antioxidative molecules may make pancreatic β-cells easily destroyed by oxidative stress under autoimmune attack in type 1 diabetes as well as under chronic hyperglycemia and/or increased insulin demand due to insulin resistance in type 2 diabetes, contributing to increased susceptibility to both type 1 and type 2 diabetes. *Nxn* is therefore a candidate gene for both type 1 and type 2 diabetes as well as STZ-induced diabetes.

To study the possibility of whether or not mutation of *Nxn* is responsible for *Idd4* effect, a susceptibility gene for type 1 diabetes on Chr 11, we compared the sequences between NOD and B6, which were used to map *Idd 4* [11]. Comparison of the sequence of *Nxn* in the NOD mouse with the published sequence in B6 revealed that the two sequences are identical, indicating that the sequence variation in *Nxn* determined in the present study is unlikely to be a candidate for *Idd4* in the NOD mouse. Since a susceptibility gene for type 2 diabetes, *Nidd1n*, was mapped in crosses of NSY with C3H mice [23], we determined the nucleotide sequence of *Nxn* in the C3H mouse as well as in the NSY mouse. The nucleotide sequence for *Nxn* in the NSY mouse was different from the published sequence of C57BL/6, but identical to that in the C3H mouse. The identical sequence in NSY and C3H indicates that allelic variation

of *Nxn* is unlikely to be a candidate for *Nidd1n* or the STZ-sensitivity gene on Chr 11 in the NSY mouse.

In summary, the NSY mouse, which is a sister strain of the STZ-sensitive NOD mouse, was shown to be susceptible to STZ-induced diabetes, indicating that the two strains, NOD and NSY, derived from the same outbred colony share the same characteristic of intrinsic vulnerability of β-cells to STZ. Conversion of STZ-resistant C3H mice to STZ-sensitive by introgression of the NSY-derived Chr 11 clearly indicates that STZ-sensitivity of the NSY mouse is determined, at least in part, by Chr 11 of the NSY mouse. The fact that STZ-sensitivity of the consomic strain was weaker than that of NSY mice and stronger than that of C3H mice suggests that susceptibility to STZ-induced diabetes in the NSY mouse is controlled by multiple genes, one of which is located on Chr 11. The sequence of the gene for nucleoredoxin, a candidate gene for type 1, type 2, and STZ-induced diabetes, was determined in four strains of mice and nucleotide substitutions were identified at two positions, but these substitutions did not correlate with susceptibility to diabetes, suggesting that genes other than *Nxn* on Chr 11 may be responsible for susceptibility to diabetes.

Acknowledgments

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