Original Article

The Idd4 Locus Displays Sex-Specific Epistatic Effects on Type 1 Diabetes Susceptibility in Nonobese Diabetic Mice

Evgueni A. Ivakine,[1,2] Steven M. Mortin-Toth,[1] Omid M. Gulban,[1] Aneta Valova,[1] Angelo Canty,[3] Christopher Scott,[1] and Jayne S. Danska[1,2,4]

The nonobese diabetic (NOD) mouse recapitulates many aspects of the pathogenesis of type 1 diabetes in humans, including inheritance as a complex trait. More than 20 *Idd* loci have been linked to type 1 diabetes susceptibility in NOD mice. Previously, we used linkage analysis of NOD crossed to the nonobese diabetes-resistant (NOR) strain and NOD congenic strains to map susceptibility to both spontaneous and cyclophosphamide-accelerated type 1 diabetes to the *Idd4* locus on chromosome 11 that displayed a sex-specific effect on diabetes susceptibility. Here, we elucidate the complex genetic architecture of *Idd4* by analysis of congenic strains on the NOD and NOR backgrounds. We previously refined *Idd4.1* to 1.4 Mb and demonstrated an impact of this interval on type 1 interferon pathways in antigen-presenting cells. Here, we identify a second subregion, the 0.92 Mb *Idd4.2* locus located telomeric to *Idd4.1*. Strikingly, *Idd4.2* displayed a sex-specific, epistatic interaction with *Idd4.1* in NOR.NOD congenic females that was not observed in syngenic males. *Idd4.2* contains 29 genes, and promising candidates for the *Idd4.2* effect on type 1 diabetes are described. These data demonstrate sex-dependent interaction effects on type 1 diabetes susceptibility and provide a framework for functional analysis of *Idd4.2* candidate genes. *Diabetes* 55:3611–3619, 2006

Type 1 diabetes is a complex multifactorial disease of autoimmune etiology in which susceptibility is conferred by an interaction between multiple genetic loci and environmental factors. Identification of genes responsible for type 1 diabetes susceptibility is essential to understand pathogenic mechanisms, to provide markers for preclinical detection of the disease process, and to identify specific targets for therapeutic intervention. The nonobese diabetic (NOD) mouse recapitulates many features of human type 1 diabetes, providing an excellent model for aspects of regulation of the diabetogenic process. More than 20 insulin-dependent diabetes (*Idd*) loci have been identified in the diabetes-prone NOD mouse (1,2), and human studies (3,4) have revealed similarly complex inheritance. To date, six have been characterized on a molecular level in humans or NOD mice. The major susceptibility locus, *Idd1* (mice) and *IDDM1* (humans), is the major histocompatibility complex class II haplotype controlling antigen presentation to $CD4^+$ T-cells (2). An allele of β2-microglobulin residing in the NOD *Idd13.1* locus and varying by one amino acid can modulate type 1 diabetes susceptibility, suggesting that variants affecting major histocompatibility complex class I function are also important in disease (5). Two regulators of T-cell activation, *CTLA4* and *PTPN22*, have been shown to be type 1 diabetes susceptibility genes in the NOD (6) and human (7,8) models. The central role of macrophage function in type 1 diabetes pathogenesis recently was underscored by evidence that NRAMP1, an endosomal ion transporter operative in resistance to intracellular bacteria, is probably responsible for the strong effect of the *Idd5.2* locus on type 1 diabetes in NOD mice (9). Strong type 1 diabetes association with the number of variable nucleotide tandem repeats in the insulin gene promoter has also been associated with diabetes risk in humans (10). While mice have two insulin genes and lack variable nucleotide tandem repeat elements, insulin has been identified as an important autoantigen in NOD mice (rev. in 11). Identification of the type 1 diabetes genes by linkage analysis in human populations has been hampered by the low penetrance of the diabetes phenotype. Congenic mapping in the NOD mouse has provided confirmation of statistical evidence of linkage and refined multiple intervals to a practical size for molecular genetic analysis. Using this approach, many linkage peaks have been shown to reflect closely linked susceptibility loci: *Idd3*, *Idd10*, *Idd17*, and *Idd18* on chromosome 3 (rev. in 12); *Idd5.1*, *Idd5.2*, and *Idd5.3* on chromosome 1 (13,14); *Idd9.1*, *Idd9.2*, and *Idd9.3* on chromosome 4 (15); and *Idd13.1* and *Idd13.2* on chromosome 2 (16). In some cases, genetic interactions between loci situated on the same chromosome displayed additive (*Idd9.1* and *Idd9.2*) (15) or epistatic (*Idd3* and *Idd10*) (17) effects on the diabetes phenotype. The proximity of these susceptibility variants likely reflects enhanced probability of their detection by linkage. As described here, closely linked variants may exert coordinate effects on type 1 diabetes.

To focus effort on type 1 diabetes susceptibility loci predicted to have a strong effect on disease, we compared the NOD mouse with the closely related nonobese diabe-

From the [1]Program in Developmental Biology, Hospital for Sick Children, Toronto, Ontario, Canada; the [2]Department of Immunology, University of Toronto, Toronto, Ontario, Canada; the [3]Department of Mathematics and Statistics, McMaster University, Hamilton, Ontario, Canada; and the [4]Department of Medical Biophysics, Institute of Medical Sciences, University of Toronto, Toronto, Ontario, Canada.

Address correspondence and reprint requests to Jayne S. Danska, 101 College St., MaRS East Tower, Rm. 14-313, Toronto, ON, M5G 1L7 Canada. E-mail: jayne.danska@sickkids.ca.

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CY-T1D, cyclophosphamide-accelerated type 1 diabetes; SNP, single nucleotide polymorphism.

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TABLE 1
Sequencing primers used in this study

Gene	Primer F	Primer R
Nos2-cDNA-A	CCCACGGGACACAGTGTCACTGGTT	TTCCTTTGTTACAGCTTCCAGCCTGGC
Nos2-cDNA-B	GAGCTCCTGCCTCATGCCATTGAGTTC	GGTGCCCATGTACCAACCATTGAAG
Nos2-cDNA-C	GCTCGGGTTGAAGTGGTATGCACTGC	GTCCATGCAGACAACCTTGGTGTTGAAG
Nos2-cDNA-D	GGTCAGAGCCACAGTCCTCTTTGCTACTGAG	TAGCTGGGCCCTCGGCTGCC
Nos2-cDNA-E	CCATGAGGCTGAAATCCCAGCAGAATC	ACTGACACTTCGCACAAAGCAGGGC
Nos2-cDNA-F	CGAGATGGTCAGGGTCCCCTGC	AGTGCCAGAAGCTGGAACTCTGGGC
Nos2 4000-5′ regulatory	CTTCACTGCTGAGTTATCTCACCAGC	GTTTTTGGTCAACAGTTGGGAGATTC
Nos2 7000-5′ regulatory	CCTTCGCTTCTAAAGCCATCTTGTC	ACCTTCCTTAAGGGGCTATGCATTC
Nos2 8000A-5′ regulatory	CCTGTCTACTTCGACCCATAACCACTC	GGGTTCAGCCAGGTATGCCTCTAAG
Nos2 8000B-5′ regulatory	GGACAGAGCATTCACCATCTGGTC	GAGATCTGACTCCCTATTCTGGAGTGTC

tes-resistant (NOR) strain. NOD and NOR mice share all *Idd* loci identified to date, except *Idd4*, *-5*, *-9*, *-11*, and *-13* (14,16,18), which are sufficient to protect NOR animals from both spontaneous and cyclophosphamide-accelerated type 1 diabetes (CY-T1D). Cyclophosphamide accelerates type 1 diabetes onset from months to <4 weeks with cumulative incidence of the disease of 80–100% (19). Like the spontaneous disease, CY-T1D pathogenesis is dependent upon both macrophages/dendritic cells and T-cells (rev. in 20). Type 1 diabetes–resistant strains (21), including NOR mice (22), remain largely disease free following cyclophosphamide treatment. The strong sex bias in spontaneous type 1 diabetes incidence (~85% in females vs. 15–40% in males) (23) is attenuated by cyclophosphamide, which results in a high frequency of disease in both sexes (19,24).

Previously, we took advantage of cyclophosphamide accelerating effects to analyze genetic control of type 1 diabetes and to gain insight into the sex-biased incidence of the disease. We showed that genetic control of differential CY-T1D susceptibility between NOD and NOR mice was controlled by *Idd4*, *-5*, and *-9* loci (24). The *Idd4* locus displayed sex-specific effects on CY-T1D: NOD.NOR-*Idd4* congenic females displayed disease protection, while syngenic males remained susceptible. Subsequently, we reported fine genetic mapping of this locus in male congenic mice on the NOR background to a 1.4-Mb region (25). Here, we identified a second locus *Idd4.2*, located just telomeric to the previously positioned *Idd4.1* (25). The disease risk conferred by NOD *Idd4.2* was specific to females, in which the interval displayed epistatic interaction with *Idd4.1*, which was not observed in males. We refined *Idd4.2* to a 0.92-Mb interval containing 29 genes. These data provide high-resolution analysis of the *Idd4* locus supporting locus-locus interactions and the sex-specific behavior of autosomal loci in type 1 diabetes.

RESEARCH DESIGN AND METHODS

All mice used in this study were maintained in a specific pathogen-free facility at the Hospital for Sick Children. Spontaneous diabetes incidence at age 6 months in NOD/Jsd animals is 83% in females and 35% in males. All procedures performed on these mice followed the guidelines of the institutional animal care committee.

Genomic DNA preparation and genotyping. Genomic DNA was prepared from tail snips with a DNA Easy kit (Qiagen) for use in PCR amplification. All microsatellite markers used in this study were amplified as follows: 10 cycles: 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C followed by 35 cycles: 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, in 1.5 mmol/l $MgCl_2$. The amplification products were visualized following electrophoresis through 2% NuSieve (American Bioanalytical) and 2% agarose (Life Technologies) gels.

Generation of *Idd4* subcongenic mice. Generation of the NOR.NOD-*Idd4* (R1-R4) congenic strains was previously described (25). A novel NOR.NOD-*Idd4* (R5) was generated using the same approach. Previously described NOR.NOD-*Idd4* mice (24) were backcrossed to NOR, their pups were intercrossed, and the resulting progeny genotyped for D11Mit74, D11Mit340, D11Mit230, D11Mit135, D11Mit217, D11Mit310, D11Mit164, D11Mit157, D11Mit177, D11Mit4, D11Mit368, D11Mit30, D11Mit90, D11Mit364, D11Mit219, D11Mit322, and D11Bhm149. NOR.NOD-*Idd4* (R5) subcongenic strain was generated by backcrossing informative recombinant to NOR and intercrossing the progeny to fix NOD-derived subcongenic interval. Two novel Idd4 subcongenic strains on the NOD genetic background (NOR.NOD-Idd4 [R1] and NOD.NOR-Idd4 [R2]) were generated from previously reported NOD.NOR-*Idd4* mice (24) using a reciprocal strategy.

Cyclophosphamide treatment and diabetes assessment. Cyclophosphamide treatment and diabetes assessment were performed as previously described (24). The difference in diabetes incidence between parentals and congenic strains was assessed using Fisher's exact test in statistical package SPSS for PC version 8.01 (SPSS, Chicago, IL). Analysis of differences in CY-T1D incidence and latency among strains was determined with Kaplan-Meier life tables and computation of mean time to type 1 diabetes onset ± SD.

Generation and testing of novel markers. Novel microsatellite markers were generated as previously described (25). To identify informative markers, NOD, NOR, DBA/2J, and C57BL/6, DNA was amplified for 35 cycles with the following conditions: 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C in 1.5 mmol/l $MgCl_2$. The amplification products were electrophoresed through either 2% NuSieve (American Bioanalytical) and 2% agarose (Life Technologies) or 8% acrylamide gels. Novel microsatellite markers used in this study are presented in online appendix Table 1 (available at http://diabetes.diabetesjournals.org).

Nos2 gene sequencing. The coding and regulatory regions of the *Nos2* gene were compared between NOD and NOR strains by direct sequencing. cDNA prepared from lipopolysaccharide- and γ-interferon–activated bone marrow–derived macrophages/dendritic cells (25) was used as a PCR template under the following conditions: 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C in 1.5 mmol/l $MgSO_4$ for 30 cycles, using high-fidelity *Taq* polymerase (Invitrogen, Carlsbad, CA). The regulatory regions of the *Nos2* gene were sequenced from genomic DNA under the same conditions. Sequencing primers, designed with Primer Express 1.5 software (Applied Biosystems, Foster City, CA), are listed in Table 1. PCR products were sequenced on both strands at the Centre for Applied Genomics (Hospital for Sick Children, Toronto, ON, Canada). Sequence files were compared with Lasergene software (DNASTAR, Madison, WI).

Analysis of the genomic structure of the Idd4.2 locus. Single nucleotide polymorphisms (SNPs) between NOD/LtJ, DBA/2J, and C57BL/6J strains were examined from the Perlegen (available at http://mouse.perlegen.com/mouse) and Broad Institute (available at http://www.broad.mit.edu/mouse) databases. Perlegen reported that SNPs were positioned on the National Center for Biotechnology Information sequence build 34 and Broad Institute SNPs on build 33. SNPs from the two sources were extracted using Pearl script; rows that contained an N (unresolved nucleotide) within these datasets were removed from the analysis. The remaining filtered data, 85 SNPs from the Broad Institute and 639 SNPs from Perlegen, were used for comparison between strains.

Testing for sex-by-strain interaction effect in CY-T1D. To test for sex-by-strain interaction effects, we performed a logistic regression analysis in which the log odds were modeled as a linear function of the covariates, as previously described (24,26). A significant sex-specific effect of a locus was assumed if the ratio of the odds of diabetes in the parental strain and the odds of diabetes in the congenic strain were different for the two sexes. We fitted a logistic regression model with three terms: a main effect for sex, a main effect for strain, and a sex-by-strain interaction term. We also fitted a second model without the interaction term that assumed that the log odds ratio was the same in both sexes. The test statistic for the significance of the sex-

dependent effect was twice the difference in log likelihoods between these two models, with a χ^2 distribution with a single degree of freedom if the null hypothesis of no interaction is true. The *P* values presented were obtained using this χ^2 distribution.

RESULTS

The telomeric boundary of genetic nonidentity between NOR and NOD on chromosome 11. We previously mapped *Idd4* to a telomeric portion of chromosome 11 where the NOR genome is C57BLKS/J (BKs)-derived (24). To guide our mapping efforts, we first mapped the telomeric boundary of BKs-derived DNA on NOR chromosome 11. Multiple novel microsatellite markers were tested for the polymorphism between NOD and NOR strains (online appendix Table 1) and placed this boundary between BKs- and NOD-derived DNA between the D11Jyh1081 and D11Mit33 spaced 0.9 Mb apart. However, the telomeric BKs-derived segment on NOR chromosome 11 was not identical to either of the reported DBA/2 and C57BL/6 progenitor strains of NOR (27), suggesting an additional genomic contribution from an unknown strain (online appendix Table 1). Our results are consistent with a recent SNP mapping study of the BKs strain, which suggested that ~9% of its genome differed from both DBA/2 and C57BL/6 (28).

Exclusion of Nos2 as an Idd4 candidate gene. Refinement of the telomeric boundary of *Idd4* confirmed that inducible nitric oxide synthase (*Nos2*) resides within this region (78646511 bp). Multiple studies have demonstrated that nitric oxide can damage pancreatic β-islets (rev. in 29), suggesting that activity/expression of NOS2 may contribute to type 1 diabetes susceptibility. To investigate this possibility, we sequenced coding and regulatory regions of *Nos2* in NOD and NOR strains. The regulatory regions were selected based upon the PipMaker algorithm for phylogenetic conservation (30), applied 10 kb upstream of the *Nos2* transcription start site gene using mouse and human Ensembl sequence. This analysis revealed four phylogenetically conserved elements: *1*) 1,800-bp region immediately upstream of the gene previously identified as a promoter region conferring lipopolysaccharide and interferon responsiveness (31), *2*) a 600-bp region located 4,000 bp upstream, *3*) a 150-bp region 7,000 bp upstream, and *4*) a 1,000-bp region 8,000 bp upstream. The sequence of the minimal promoter region revealed no variations between NOD and NOR strains. Similarly, no differences in sequence were found in either coding or the predicted regulatory regions (data not shown). However, these data could not exclude the presence of polymorphisms between NOD and NOR in other unidentified regulatory regions. Genetic analysis of informative congenic strains was then used to exclude *Nos2* as a candidate gene (see below).

CY-T1D in NOR.NOD-Idd4 subcongenic mice. Previously, we demonstrated that NOR.NOD-*Idd4* mice are susceptible to CY-T1D (24) and refined the *Idd4.1* locus to a 1.4-Mb interval using congenic strains (25). The latter study was performed in male mice due to the higher penetrance of the type 1 diabetes susceptibility in this sex. We generated an additional congenic strain (NOR-R5) with a recombination point between D11Mit90 and D11Mit364. Fine mapping of this strain using novel microsatellite markers placed the recombination event within 200 kb telomeric to the boundary of *Idd4.1* between markers D11Gul2700 and D11Gul2721 (data not shown). When male and female NOR-R5 mice were assessed for CY-T1D and compared with the parental NOR strain, a striking sex difference in disease susceptibility was observed. In contrast to all other *Idd4* recombinants on the NOR background, where CY-T1D susceptibility was equivalent in both sexes compared with NOR parental mice (25; Fig. 1), cyclophosphamide treatment of NOR-R5 mice had a distinct effects depending on the sex of the mice. NOR-R5 males progressed to diabetes (32% type 1 diabetes, $n = 60$; $P = 0.011$ vs. NOR; 11% type 1 diabetes, $n = 36$), whereas females were CY-T1D resistant ($P = 0.76$ vs. NOR). Analysis of NOR-R5 also indicated that our previously reported strong effect of NOD-derived *Idd4.1* locus in males (25) was insufficient to confer CY-T1D in females. Taken together with the observation that NOR-R3 females carrying a longer NOD-derived segment were susceptible to CY-T1D ($P = 0.011$ vs. NOR), these data suggested that NOD *Idd4*-mediated CY-T1D susceptibility in females required at least two genes, *Idd4.1* and *Idd4.2*. Enhanced susceptibility to CY-T1D in NOR-R1 and NOR-R3 congenic females was not associated with a change in time to disease onset (Kaplan-Meier analysis data not shown; mean time to onset ± SD in days: 21.00 ± 5.44 to 24.91 ± 3.78). Based upon these NOR recombinant congenic strains, *Idd4.2* was located in a 5.6-Mb interval, between D11Gul2700 and D11Mit322.

CY-T1D in NOD.NOR-Idd4 recombinant congenic mice. Having observed that NOD.NOR-*Idd4* females, but not males, were protected from CY-T1D (24), we generated two *Idd4* subcongenic strains on the NOD background and analyzed them for susceptibility to CY-T1D (Fig. 2). Compared with NOD mice, NOD-R1 females were not protected from disease ($P = 0.18$), whereas NOD-R2 animals were CY-T1D resistant ($P = 0.001$). Decreased incidence of CY-T1D in NOD-R2 congenic females was not associated with a change in time to disease onset (Kaplan-Meier analysis data not shown; mean time to onset ± SD in days: 19.00 ± 5.82 to 20.05 ± 6.20). Since NOD-R1 were not distinguishable from NOD females, the region required to confer CY-T1D protection resided telomeric to D11Mit219 consistent with a second locus, *Idd4.2*.

Fine mapping of Idd4.2 locus. To refine the position of *Idd4.2*, we utilized the Celera and Ensembl databases of mouse genome sequence to define a novel set of microsatellite markers (see RESEARCH DESIGN AND METHODS). PCR amplicons containing the microsatellite repeats were tested for polymorphism between NOD and NOR strains (online appendix Table 1), and informative markers were used to generate a high-resolution map of the recombination boundaries in the relevant congenic strains. Analysis of the NOD-R1 strain with new markers between D11Mit219 and D11Mit322 positioned the centromeric boundary of *Idd4.2* within a 6-kb interval defined by D11Gul4 and D11Gul5 (Table 2). The telomeric boundary, identified by genotyping the NOR.NOD-R3 strain, was placed in a ~500-kb interval between the D11Gul33 and D11Gul80. These results refined *Idd4.2* to a 0.92-Mb region including the boundary regions containing 29 genes (Table 3). To date, further refinement of the 500-kb *Idd4.2* telomeric boundary has been limited by the absence of polymorphic markers between NOD and NOR strains in this region.

Genomic architecture of the Idd4.2 locus. Having refined the *Idd4.2* locus to 0.92 Mb, we then considered the 29 genes in the interval (Table 3). Of these, 14 are olfactory receptors located within the 0.7-Mb telomeric portion of the locus. Due to the restricted tissue expres-

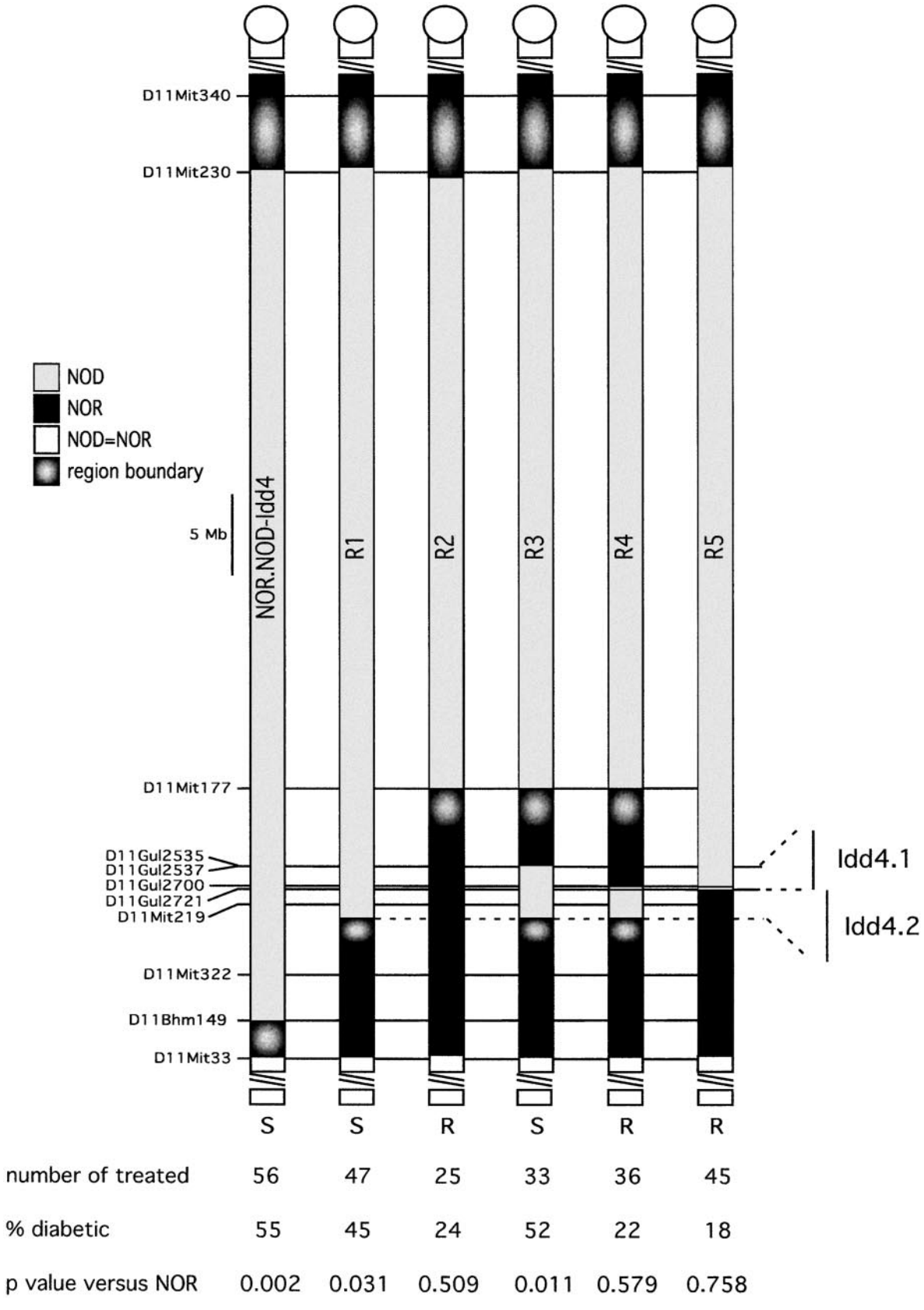

FIG. 1. Identification of the *Idd4.2* locus. Female mice of different NOR.NOD-*Idd4* subcongenic strains were tested for CY-T1D as described in RESEARCH DESIGN AND METHODS. The strains were genotyped for indicated microsatellite markers to determine locations of NOD-derived (gray) and NOR-derived (black) regions. Unresolved boundaries between NOD- and NOR-derived intervals are depicted in shaded gray ovals, and regions identical by descent between parental strains are shown in white. Percentage of diabetic females for each strain, as well as a Fisher's exact test *P* values versus parental NOR strain (24%, $n = 33$) as a measure of resistance (*R*) or susceptibility (*S*) to CY-T1D, are illustrated.

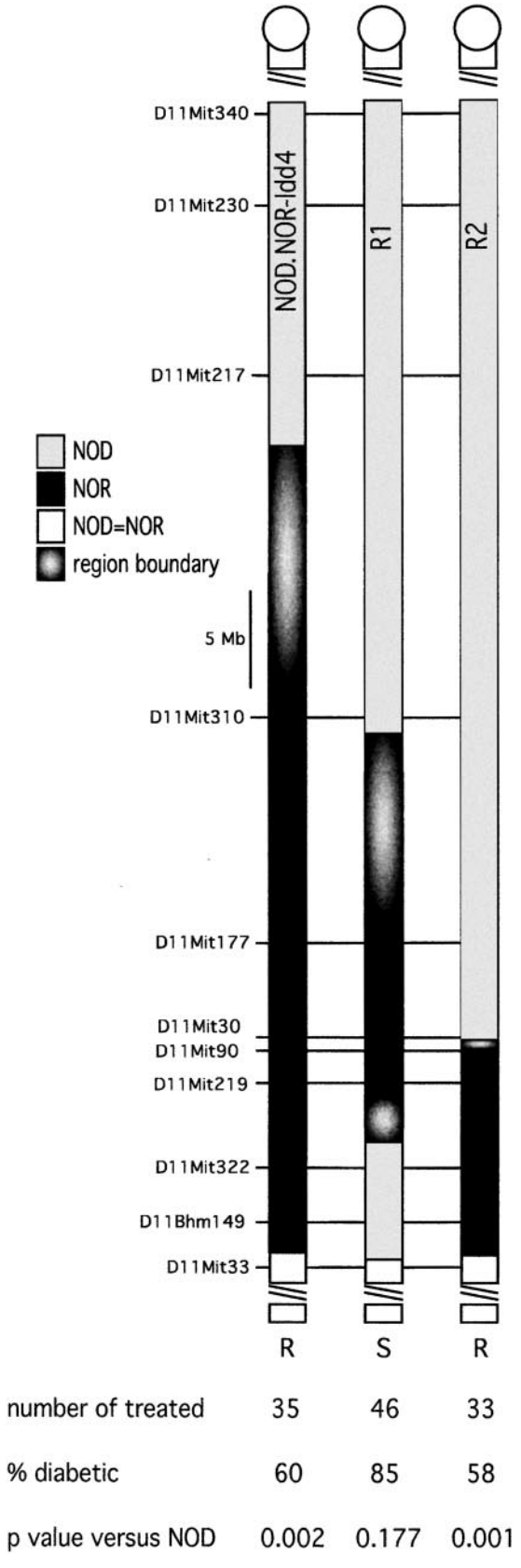

FIG. 2. CY-T1D in females of *Idd4* subcongenic strains on the NOD genetic background. Percentage of diabetic females for each strain, as well as Fisher's exact test *P* values versus parental NOD strain (92%, $n = 26$) as a measure of resistance (*R*) or susceptibility (*S*) to CY-T1D, are illustrated.

sion pattern of these genes (olfactory epithelium), they were viewed as low priority as candidate genes for *Idd4.2*. To better appreciate the genomic structure of the locus, we compared the NOD, C57BL/6, and DBA/2 strain SNPs from the Perlegen and Broad Institute databases (online appendix Tables 2 and 3). A summary of these comparisons is provided in Table 4.

The marker distribution across *Idd4.2* is different between the Broad Institute and Perlegen datasets because the latter lacked SNPs within the ~0.7-Mb telomeric region of the locus (online appendix Table 2). Indeed, the majority of Broad Institute markers within *Idd4.2* that were not polymorphic between NOD, DBA/2, and B6 were clustered within this 0.7-Mb region. The paucity of informative markers in this portion of the locus limited further refinement of the *Idd4.2* telomeric boundary. In the centromeric portion of the locus, the majority of the markers was identical between NOD and B6 and differed from the DBA/2 strain. The genomic diversity at *Idd4.2* is consistent with our previous analyses of NOD genomic sequence of the *Idd4.1* locus revealing remarkable similarity between NOD and B6 strains (25). Thus, NOD and B6 appear to share an extended ancestral haplotype spanning *Idd4.1*, the *Idd4.1–Idd4.2* interlocus region, and the centromeric portion of the *Idd4.2*. Broad Institute SNP data for C57BLKS/J, the other progenitor of NOR mice (27), showed that all *Idd4.2* SNPs were identical between C57BLKS/J and DBA/2 (online appendix Table 3), indicating that, as for *Idd4.1*, NOR-derived protection from CY-T1D at *Idd4.1* and *Idd4.2* is mediated by DBA/2-derived alleles.

Sex-specific behavior and epistatic interactions of the Idd4.1 and Idd4.2 loci. The *Idd4* locus displays sex-specific behavior and consists of at least two loci: *Idd4.1* and *Idd4.2* (25; this study). Given the sex-dependent phenotype of NOR-R5 mice, the NOD *Idd4.1* locus alone conferred CY-T1D in males but not in females. We compared NOR versus NOR-R5 and NOR versus NOR.NOD-*Idd4* strains to determine whether the effects of the genotype differed by sex. A χ^2 likelihood ratio test was done to determine whether an interaction term was needed to explain these effects. Comparison of NOR-R5 and NOR mice showed a significant sex-by-strain interaction effect (Fig. 3; $P = 0.038$), supporting the sex-specific behavior of alleles at the *Idd4.1* locus. In the NOR-R4 and NOR-R5 congenic strains, neither NOD *Idd4*.1 nor *Idd4.2* alone was sufficient to confer CY-T1D risk in females (Fig. 1). When NOD alleles at *Idd4.1* and *Idd4.2* were combined in the NOR-R3 strain, females did acquire significant susceptibility to CY-T1D. These findings are consistent with an epistatic interaction between NOD alleles at *Idd4.1* and *Idd4.2* that together confer risk for CY-T1D development in females. While NOD *Idd4.1* alone increased CY-T1D susceptibility in NOR congenic males, comparison between cohorts of male NOR-R5 (19 type 1 diabetic, 41 non–type 1 diabetic) and NOR.NOD-*Idd4* (24 type 1 diabetic, 23 non–type 1 diabetic) mice showed a difference (two-sided $P = 0.0423$), consistent with modest additive effects of NOD alleles at *Idd4.1* and *Idd4.2*.

DISCUSSION

We present a high-resolution analysis of the *Idd4* diabetes susceptibility locus and document features of the complex genetic architecture of the region. *Idd4* was found to consist of two regions, *Idd4.1* and *Idd4.2* (25; this study).

TABLE 2
High-resolution mapping of the *Idd4.2* locus

Marker	Position	NOR-R1	NOR-R3	NOD-R1			
D11Mit364	71958448	NN	NN	BB			
D11Mit219	72044629	NN	NN	BB			
D11Jyh522	72136258	NN	NN	BB			
D11Jyh548	72236138	NN	NN	BB			
D11Jyh582	72568517	NN	NN	BB			
D11Jyh598	72706982	NN	NN	BB			
D11Gul4	72723423	NN	NN	BB			
D11Gul5	72728763	*NN*	*NN*	*NN*			
D11Jyh608	72760171	*NN*	*NN*	*NN*			
D11Jyh611	72807577	*NN*	*NN*	*NN*			
D11Jyh616	72845534	*NN*	*NN*	*NN*	**0.41Mb**		**0.92Mb**
D11Jyh618	72848262	*NN*	*NN*	*NN*			
D11Jyh629	72971220	*NN*	*NN*	*NN*			
D11Gul14	72986906	*NN*	*NN*	*NN*			
D11Gul33	73133966	*NN*	*NN*	*NN*			
D11Gul80	73645126	NN	BB	NN			
D11Gul96	73894486	BB	BB	NN			
D11Jyh306	73988252	BB	BB	NN			
D11Jyh164	75329443	BB	BB	NN			
D11Jyh134	75554436	BB	BB	NN			
D11Jyh107	75860300	BB	BB	NN			
D11Jyh104	75961098	BB	BB	NN			
D11Jyh96	76227476	BB	BB	NN			
D11Mit322	76304982	BB	BB	NN			
D11Bhm149	77408880	BB	BB	NN			
D11Mit33	**81507909**	**NN**	**NN**	**NN**			

For the fine mapping of the *Idd4.2* locus, we genotyped informative congenic strains (NOR-R1, NOR-R3, and NOD-R1) with microsatellite markers polymorphic between NOD and NOR strains. All three strains are CY-T1D susceptible compared with the corresponding parental strain (Figs. 1 and 2). The genotype at each marker is shown as either NN (NOD derived) or BB (NOR derived). Position of the each marker is according to the Ensembl database (http://www.ensembl.org/mouse). Sizes of the minimal and the most conservative estimates of the *Idd4.2* locus are also indicated. Genotypes of the mice within minimal interval region are shown in bold font. The genotype of the NOR-R4 strain was not included in this table because it was identical to the NOR-R3, most likely reflecting a common genetic ancestor for these two strains.

Strikingly, both *Idd4.1* and *Idd4.2* displayed sex-specific effects on CY-T1D susceptibility. NOD-derived *Idd4.1* was sufficient to confer enhanced CY-T1D frequency in NOR congenic males, whereas both NOD-derived *Idd4.1* and *Idd4.2* were required in females, consistent with a sex-specific epistatic interaction between these loci. Theses results complement our earlier report that the *Idd4* locus behaved in a sex-specific manner (24). In contrast to most other autoimmune diseases, type 1 diabetes is often cited as an organ-specific autoimmune disease without a female sex bias (rev. in 32). While parity between sexes is generally observed in higher-incidence populations, lower-incidence groups including African Americans and Mexicans show a male bias, while Asians display a female bias (33). Furthermore, there is evidence that the peak age at onset occurs earlier in girls than in boys (34). A recent study examined association between an SNP in the estrogen-responsive *IL6* promoter (174G/C) and age at onset in boys and girls. Homozygosity for the 174C genotype was significantly less frequent in females diagnosed after 10 years of age than those diagnosed at younger ages, while no 174G/C genotype difference was observed in males stratified for age at onset (35). Thus, in the context of evidence that many autoimmune disease including type 1 diabetes are influenced by sex, the *Idd4* locus in the NOD model provides a system to examine genetic mechanisms that underlie the sexual dimorphism in type 1 diabetes.

Idd4 was first identified in a genome-wide linkage analysis of (NOD × B10.$^{H\text{-}2g7}$) × NOD animals, as a 30-cM region on chromosome 11 linked to early-onset type 1 diabetes (36). Linkage of the *Idd4* locus to spontaneous type 1 diabetes was later confirmed in NOD.B6 congenic animals, where the strongest diabetes resistance mapped to a 20-Mb interval between the D11Mit30 and D11Mit41 markers (37). Both the *Idd4.1* and *Idd4.2* loci described in our study reside within this interval. Grattan et al. (37) also proposed existence of the two loci within *Idd4*. However, the resolution provided by these NOD.B6-*Idd4* congenic strains limit determination of the precise positional relationship between their *Idd4* loci and those we have reported (25; this study).

Which genes within *Idd4.2* might regulate susceptibility to CY-T1D and how could they explain the sex-dependent behavior of this locus? The 14 olfactory receptor genes in the *Idd4.2* region (Table 3) displayed expression restricted to the olfactory epithelium, making them low priority candidates for type 1 diabetes susceptibility. Among the remaining genes two purinergic receptors, *P2rx1* and *P2rx5*, and an integrin α-E (*Itgae*) have links to immune functions.

P2RX1 and P2RX5 belong to a family of nonselective cation channel proteins activated by extracellular ATP. Interestingly, *P2rx1*-deficient males, but not females, displayed reduced fertility compared with the wild-type counterparts, suggesting that function of this receptor in some tissues depends on sex (38). P2RX5 expression is highest in brain and the immune system, implicating a possible role in immune responses (39). Another member of the

TABLE 3
Genes within the *Idd4.2* region

Ensembl gene ID	Start	Gene name	Gene description
D11Gul4	72723423		
ENSMUSG00000020787	72724801	P2rx1	P2X purinoceptor 1 (ATP receptor)
D11Gul5	72728763		
ENSMUSG00000020785	72744702	Camkk1	Calcium/calmodulin-dependent protein kinase kinase 1 α
ENSMUSG00000020783	72773539	1200014J11Rik	ELG protein
ENSMUSG00000005947	72816242	Itgae	Integrin α-E precursor
ENSMUSG00000050107	72861656	Gsg2	Germ cell–Specific gene 2
ENSMUSG00000005950	72886184	P2rx5	Purinergic receptor P2X5
ENSMUSG00000047260	72901192	0610009E20Rik	Null
ENSMUSG00000040158	72902742	Tax1bp3	TAX1 (Human T-cell leukemia virus type I) binding protein 3
ENSMUSG00000005949	72908792	Ctns	Cystinosin
ENSMUSG00000005951	72925141	Carkl	Carbohydrate kinase–like protein
ENSMUSG00000005952	72963879	Trpv1	Transient receptor potential cation channel, subfamily V, member 1
ENSMUSG00000043029	72993279	Trpv3	Transient receptor potential cation channel, subfamily V, member 3
ENSMUSG00000020774	73030647	Aspa	Aminoacylase 2
ENSMUSG00000069825	73056811	Q5SV06_mouse	Testes development related by homology
ENSMUSG00000062128	73079414	Olfr20	Olfactory receptor 20
ENSMUSG00000063881	73100410	Olfr376	Olfactory receptor 376
ENSMUSG00000069823	73120735	Olfr1	Olfactory receptor 1
D11Gul33	73133966		
ENSMUSG00000055971	73150696	Olfr378	Olfactory receptor 378
ENSMUSG00000061764	73178934	Olfr380	Olfactory receptor 380
ENSMUSG00000060588	73211546	Olfr381	Olfactory receptor 381
ENSMUSG00000059470	73314457	Olfr385	Olfactory receptor 385
ENSMUSG00000060335	73328241	Olfr386	Olfactory receptor 386
ENSMUSG00000060630	73415339	Q5SWG7_mouse	Novel krab box–containing protein (fragment)
ENSMUSG00000069819	73502052	Olfr389	Olfactory receptor 389
ENSMUSG00000069818	73512599	Olfr390	Olfactory receptor 390
ENSMUSG00000061984	73539801	Olfr392	Olfactory receptor 392
ENSMUSG00000064228	73572203	Olfr393	Olfactory receptor 393
ENSMUSG00000056921	73613097	Olfr394	Olfactory receptor 394
ENSMUSG00000062186	73632211	Olfr395	Olfactory receptor 395
D11Gul80	73645126		

Gene name, description, chromosomal location, and unique identifier are shown according to the Ensembl database (http://www.ensembl.org). D11Gul markers used to identify boundaries of the *Idd4.2* locus are also shown.

P2X receptor family (P2RX7) has been directly implicated in response to infection, as macrophages from *P2rx7*-deficient mice were unable to kill intracellular bacteria (40). *P2rx7* deficiency also inhibited secretion of the proinflammatory cytokine interleukin-1 resulting in greater resistance to collagen-induced arthritis in a mouse model (41). P2X receptors form channels as multimers of several subunits, and both homomeric and heteromeric receptors have been described following ectopic expression in cell lines (rev. in 42). One speculation is that either P2RX1 or P2RX5 (or both) may form heteromeric channels with P2RX7 contributing to the immune phenotypes described above. Much functional analysis is needed to appreciate the contribution of P2X channels to immune function, but current evidence makes these two genes interesting candidates for *Idd4.2*.

TABLE 4
Summary of genetic polymorphisms among B6, NOD, and DBA strains in *Idd4.2* according to the Perlegen and Broad Institute databases

Comparison	Perlegen	Broad Institute
B6 = NOD = DBA	318	63
B6 = NOD ≠ DBA	250	13
B6 = DBA ≠ NOD	44	2
NOD = DBA ≠ B6	27	7

Itgae encodes an integrin cell surface adhesion protein (CD103) thought to play an important role in mucosal immunity. CD103 is expressed on >90% of intestinal intraepithelial lymphocytes but on <2% of circulating peripheral blood T-cells (43). Recent studies (44–46) have suggested that coexpression of CD103 on $CD8^+$ T-cells marks a potent cytotoxic subset and that some $CD103^+CD8^+$ T-cells have immune regulatory functions. Consistent with its role in mucosal immunity, *Itgae*-deficient mice had reduced numbers of gut and vaginal mucosal T-cells (47) and developed a cutaneous inflammatory disorder (48). Interestingly, the majority population of dendritic cells in the lung is $CD103^+$, and their numbers are increased in mice with induced airway hyperresponsiveness, suggesting a potential role of these cells in asthma pathogenesis (49). Thus, CD103 may play multiple roles in regulating and effecting the immune response making it an attractive candidate as the type 1 diabetes susceptibility gene.

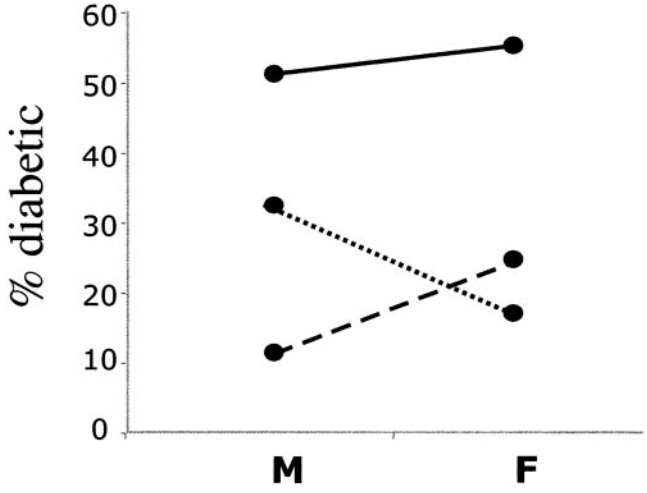

FIG. 3. Sex-specific behavior of the *Idd4.1* locus. The cumulative frequency of CY-T1D (percentage) was plotted for both males (M) and females (F) in NOR, NOR.NOD-*Idd4*, and NOR.NOD-*Idd4* (R5) strains. The sex-by-strain interaction effect was tested using logistic regression analysis as described in RESEARCH DESIGN AND METHODS for all NOR congenic versus parental strain. Only NOR.NOD-*Idd4* (R5) versus NOR comparison resulted in a statistically significant interaction effect ($P = 0.038$), providing evidence for sex-specific behavior of the *Idd4.1* locus. A NOR versus NOR.NOD-*Idd4* interaction graph ($P = 0.32$) is presented for comparison. The incidence of CY-T1D in NOR and NOR.NOD-*Idd4* males has been published elsewhere (25) and in NOD. NOR-*Idd4* (R5) was determined to be 32% ($n = 60$). For females, the incidence and numbers of animals analyzed is shown in Fig. 1. —, NOR.NOD-*Idd4*; – –, NOR; ....., NOR.NOD-*Idd4* (R5).

We present a high-resolution map of the type 1 diabetes susceptibility *Idd4* locus on mouse chromosome 11 and provide strong evidence for two loci *Idd4.1* and *Idd4.2*. Both intervals displayed dramatic sex-dependent effects. We previously confined *Idd4.1* to a 1.4-Mb interval containing 52 genes (25) and showed that the locus conveyed its strongest effect in males. In the present study, *Idd4.2* was refined to 0.92 Mb containing 29 genes. NOD alleles at *Idd4.1* and *Idd4.2* were necessary for CY-T1D susceptibility in females, and both NOD-derived *Idd4.1* and *Idd4.2* were required for the induction of CY-T1D, consistent with epistatic interaction between them. Efforts are underway to conduct DNA sequence, gene expression, and functional analyses of candidate genes at *Idd4.1* and *Idd4.2* to further elucidate the mechanisms involved in the genetic control of sex-dependent type 1 diabetes susceptibility by *Idd4*.

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