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Fine Mapping, Gene Content, Comparative Sequencing, and Expression Analyses Support *Ctla4* and *Nramp1* as Candidates for *Idd5.1* and *Idd5.2* in the Nonobese Diabetic Mouse[1]

Linda S. Wicker,[2]* Giselle Chamberlain,* Kara Hunter,* Dan Rainbow,* Sarah Howlett,* Paul Tiffen,* Jan Clark,* Andrea Gonzalez-Munoz,* Anne Marie Cumiskey,‡ Raymond L. Rosa,‡ Joanna M. Howson,* Luc J. Smink,* Amanda Kingsnorth,* Paul A. Lyons,* Simon Gregory,† Jane Rogers,† John A. Todd,* and Laurence B. Peterson‡

At least two loci that determine susceptibility to type 1 diabetes in the NOD mouse have been mapped to chromosome 1, *Idd5.1* (*insulin-dependent diabetes 5.1*) and *Idd5.2*. In this study, using a series of novel NOD.B10 congenic strains, *Idd5.1* has been defined to a 2.1-Mb region containing only four genes, *Ctla4*, *Icos*, *Als2cr19*, and *Nrp2* (neuropilin-2), thereby excluding a major candidate gene, *Cd28*. Genomic sequence comparison of the two functional candidate genes, *Ctla4* and *Icos*, from the B6 (resistant at *Idd5.1*) and the NOD (susceptible at *Idd5.1*) strains revealed 62 single nucleotide polymorphisms (SNPs), only two of which were in coding regions. One of these coding SNPs, base 77 of *Ctla4* exon 2, is a synonymous SNP and has been correlated previously with type 1 diabetes susceptibility and differential expression of a CTLA-4 isoform. Additional expression studies in this work support the hypothesis that this SNP in exon 2 is the genetic variation causing the biological effects of *Idd5.1*. Analysis of additional congenic strains has also localized *Idd5.2* to a small region (1.52 Mb) of chromosome 1, but in contrast to the *Idd5.1* interval, *Idd5.2* contains at least 45 genes. Notably, the *Idd5.2* region still includes the functionally polymorphic *Nramp1* gene. Future experiments to test the identity of *Idd5.1* and *Idd5.2* as *Ctla4* and *Nramp1*, respectively, can now be justified using approaches to specifically alter or mimic the candidate causative SNPs. *The Journal of Immunology*, 2004, 173: 164–173.

The *Idd5*[3] (*insulin-dependent diabetes 5*) region from diabetes-resistant C57BL/10 (B10) or C57BL/6 (B6) mice provides protection from type 1 diabetes (T1D) when introgressed onto the NOD background (1, 2). *Idd5* is located on mouse chromosome 1 and has been shown by congenic strain analysis to consist of at least two loci, *Idd5.1* and *Idd5.2*, positioned near the proximal and distal ends, respectively, of a 9.4-cM interval (1). *Idd5.1* was defined as a 1.5-cM B10-derived resistance interval containing the candidate genes *Casp8*, *Cflar* (FLIP), *Cd28*, and *Ctla4* (1). Diabetes protection was also observed in NOD.B6 congenic strains developed in the *Idd5.1* region (2). The proximal boundary of *Idd5.1* was more precisely defined in this second study, and *Casp8* and *Cflar* were eliminated as candidates. In addition, Lamhamedi-Cherradi et al. (2) provided evidence that another T cell regulatory molecule, ICOS (*Icos*), is encoded immediately distal of *Ctla4* and is included in the *Idd5.1* region.

The *Idd5.2* region was localized previously to a 5.1-cM portion of the *Idd5* interval using NOD.B10 congenic strains (1). Among the many candidate genes present in the large *Idd5.2* region, *Nramp1* (which has recently been renamed to *Slc11a1* which denotes the fact that the NRAMP1 protein is a memeber of the solute carrier family 11) is the most compelling because there is a known functional nonsynonymous polymorphism ($Gly^{169} > Asp^{169}$) distinguishing the NOD and B10 *Nramp1* alleles. The NOD NRAMP1 protein allotype, a divalent cation transporter in phagosomes, is wild type and mediates protection from certain infectious diseases, and the diabetes-resistant B10 allotype is a functional knockout (3). It has been proposed that NRAMP1-mediated reduction of iron in the phagosomes reduces the ability of some microbes to survive in this intracellular environment (4).

The B10-derived *Idd5* interval alone causes only a partial reduction in T1D frequency when present on the NOD background because 30% of NOD.B10 *Idd5* females are diabetic by 7 mo of age as compared with 80% of NOD females. There is nearly complete protection from diabetes (<2% disease frequency in females) when a disease-resistant *Idd5* interval is combined with a diabetes-protective allele at *Idd3* on chromosome 3 (1, 5). This combination of *Idd5* and *Idd3* also provides significant protection from the development of insulitis (1, 5) and of insulin autoantibodies (5).

In the current study, we describe congenic strains that significantly reduce the limits of the *Idd5.1* and *Idd5.2* intervals. A physical map of both intervals has been assembled based on the primary DNA sequence, and the gene content was determined. A

*Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, United Kingdom; †Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom; and ‡Department of Pharmacology, Merck Research Laboratories, Rahway, NJ 07065

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[2] Address correspondence and reprint requests to Dr. Linda S. Wicker, Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Cambridge, CB2 2XY, U.K. E-mail address: linda.wicker@cimr.cam.ac.uk

[3] Abbreviations used in this paper: *Idd*, insulin-dependent diabetes; BAC, bacterial artificial chromosome; flCTLA, full-length CTLA; flICOS, full-length ICOS; liCTLA, ligand-independent CTLA; NRAMP1, natural resistance-associated macrophage protein 1; NRP2, neuropilin-2; qPCR, quantitative PCR; sCTLA, soluble CTLA; sICOS, soluble ICOS; SNP, single nucleotide polymorphism; T1D, type 1 diabetes.

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comparison of the B6 and NOD genome sequences from the *Idd5.1* region containing the two primary candidate genes, *Ctla4* and *Icos*, showed a moderately high level of polymorphism, suggesting that the B10 and NOD alleles belong to different ancestral haplotypes. Expression of mRNAs for full-length and soluble ICOS, as well as full-length CTLA-4 (flCTLA-4) and two additional CTLA-4 isoforms, ligand-independent (liCTLA-4) and soluble (sCTLA-4), was assessed in *Idd5.1* congenic mice. The observed patterns of gene expression support the hypothesis (6) that a synonymous single nucleotide polymorphism (SNP) in exon 2 of *Ctla4* affects the expression of liCTLA-4 mRNA and may be the genetic basis of *Idd5.1*.

Materials and Methods

Congenic mouse strains and assessment of diabetes

The breeding and genotyping strategies for the development of the congenic mouse strains protected from T1D have been reviewed (7). All of the new strains developed for this study were derived from one of two diabetes-resistance strains, NOD.B10 *Idd5*R8 (R8) and NOD.B10 *Idd5*R444 (R444), described previously (1). New strains (Fig. 1) were produced by backcrossing R8 or R444 to NOD/MrkTac (Taconic Farms, Germantown, NY) and selecting for informative recombinants with subsequent fixing to homozygosity. NOD.B10 *Idd5*R974 (R974) and NOD.B10 *Idd5*R426 (R426) were derived from R8; NOD.B10 *Idd5*R444s (R444s) was derived from R444; whereas NOD.B10 *Idd5*R193 (R193) and NOD.B10 *Idd5*R46 (R46) were derived from R444s. NOD.B10 *Idd5*R467 (R467) and NOD.B10 *Idd5*R2 (R2) have been described previously (1). All congenic strains have been backcrossed to NOD 10–16 times, and nonchromosome 1 markers examined in founder lines are NOD in origin (1). Elevated urinary glucose was detected using Diastix (Miles, Elkhart, IN). Animals were considered diabetic when urinary glucose was >500 mg/dl. Diabetic mice also exhibited polydipsia, polyuria, and weight loss. The frequency of diabetes was compared between strains with the Kaplan-Meier log-rank test using Prism (GraphPad, San Diego, CA) software. The R974, R46, R2, and R444 strains are available from Taconic Farms through the Emerging Models Program (lines 974, 2193, 1092, and 1094, respectively). The derivation and disease characterization of the diabetes-resistant NOD congenic strains noted in Fig. 6 as *Idd3/5.1/5.2*, *Idd9.1/9.2/9.3*, and *Idd3/10/18* have been described (5).

Identification of new microsatellite markers and genotyping

Mice were initially genotyped by PCR using primers to the previously published microsatellite markers. To map the recombination points more precisely within these strains, further microsatellites were identified and characterized. Sequences were obtained from the EnsEMBL mouse genome sequence (http://www.ensembl.org). Repeat sequences were then identified using RepeatMasker 4-Apr-2000 version (http://ftp.genome-.washington.edu/RM/RepeatMasker.html) modified by D. Beare, Welcome Trust Sanger Institute, and L. Smink, Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, University of Cambridge. Sequences containing microsatellite repeats and ~500 bp of flanking sequence on either side were then extracted. PCR primers were designed for these microsatellite sequences using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). All primers were ordered from Sigma-Genosys (The Woodlands, TX). Forward primers labeled at the 5′ end with the fluorescent dye HEX. PCR were optimized using B10 and NOD DNA templates. Markers that were polymorphic between these two strains were used in further genotyping. All PCR were performed using Amplitaq Gold with buffer II (Applied Biosystems, Foster City, CA). When PCR product sizes were not distinguishable between B10 and NOD samples by 4% agarose gel electrophoresis, genotyping was performed using an ABI Prism 3100 genetic analyzer (Applied Biosystems). Primer sequences for novel microsatellite markers used in this study are shown in Table I.

Construction of a bacterial artificial chromosome (BAC) contig across the* Idd5.1 *interval. Markers were identified from between *D1Mit479* and *D1Mit300* according to radiation hybrid-mapping data from the MGI database (www.informatics.jax.org) and the Whitehead Institute. These markers were used to screen the RPCI-23 (B6) library and the DIL NOD BAC library for positive clones. Fingerprint data obtained by the Wellcome Trust Sanger Institute were also used to ascertain which clones overlapped with other clones in the region. A BAC contig was constructed across the originally defined *Idd5.1* interval for the B6 strain, and across portions of this same interval for NOD. Shotgun BAC sequencing was performed at the Wellcome Trust Sanger Institute.

Gene identification. The repeat masked BAC contig sequence was subjected to blast analysis against vertebrate mRNA, dbEST, and exofish blast databases. Expressed sequence tag hits were used to identify genes within the *Idd5.1* region. Mouse EnsEMBL and the orthologous region in human EnsEMBL confirmed the gene content of *Idd5.1*. The gene content of *Idd5.2* was obtained from the shotgun sequence available through mouse EnsEMBL. The orthologous human region in human EnsEMBL was also examined to confirm gene order.

Delayed-type hypersensitivity assay. Mice were immunized i.v. with 1×10^6 SRBC (TCS Biosciences, Buckingham, U.K.), and 4 days later were challenged with 5×10^8 SRBC in one hind footpad. At various times, the challenged foot was measured with calipers (Rabone Chesterman, Birmingham, U.K.). Twenty-four hours after challenge, the foot was removed and frozen immediately in liquid nitrogen. Control mice were untreated. Feet were pulverized while frozen in liquid nitrogen, homogenized, and RNA extracted in TRIzol (Invitrogen, San Diego, CA). Poly(A) RNA was prepared using a Genelute mRNA Miniprep kit (Sigma-Aldrich, St. Louis, MO) and quantitated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). PCRs were performed after reverse transcription of RNA (RT-PCR) using Superscript II (Invitrogen). Quantitative PCRs (qPCR) were performed using primers and probes purchased from Applied Biosystems. Real-time PCR product detection and analysis were performed on the TaqMan 7700 (Applied Biosystems). Results are normalized to GAPDH or mouse β_2-microglobulin (6). Primer and probe sequences for IFN-γ cDNA (8) and flCTLA-4, liCTLA-4, and sCTLA-4 cDNAs (6) have been described previously. Data between groups were analyzed for significance using an unpaired *t* test.

Icos *splice variant.* RT-PCR amplification of ICOS isoforms was performed using the 5′ primer GCACTGGAGGAGAAGACTGC and 3′ primer AGTCCATGCGTTTCCTCTGT. As well as the expected full-length isoform, other faint products were noted when the products were analyzed on an agarose gel. To test the hypothesis that one of these variants may be analogous to either the ligand-independent or soluble forms of CTLA-4, RT-PCR was performed using a forward primer (CTCACCAA GACCAAGGGAAG) in exon 2 and a reverse primer (GGATCCGTATT TCATAAATATGC) over the boundary of exons 2 and 4, a forward primer in the 5′ untranslated region (TGACACCACATCAACCTCCA) and reverse primers over the boundary of exons 1 and 3 (CAGAGCTGGGAT TCTGTTAAAAGT) and the boundary of exons 1 and 4 (GGATCCG TATTTCTGTTAAAAGTC). No RT-PCR products were seen when using the primers over the exon 1/3 or 1/4 boundaries. However, a product of the expected size was seen on a gel when using the primers in exon 2 and over the exon 2/4 boundary. This product was then sequenced and found to have the normal splice site at the end of exon 2, no exon 3, which has the transmembrane domain, then the normal beginning of exon 4. This skipping of exon 3 changes the reading frame in exon 4, resulting in a premature stop codon (see Table II). Full-length and soluble ICOS primers and probes used for qPCR are forward TACTTCTGCAGCCTGTCCAT, reverse CAGCAGAGCTGGGATTCATA, probe TGACCCACCTCCTTT TCAAGAA, and forward TACTTCTGCAGCCTGTCCAT, reverse GGA TCCGTATTTCATAAATATGC, probe TGACCCACCTCCTTTTCAA GAA, respectively.

Table I. *Microsatellite markers used to define* Idd5.2

Marker	5′ Primer	3′ Primer	Allele Size (bp) (B10/NOD)
D1pt23_8	cacaatggcacatgatcaca	gctgctgtgtgagaaacctg	179/175
D1pt252131_4	tttgtttttgagacaggcaca	cgtggtggctcatgtcttta	207/219
D1pt252131_8	taacttttgttggggctgga	gctggccagtgagctctaag	207/205

Activation of T cells in vivo and in vitro

T cells were activated in vivo by injecting 5 μg of anti-CD3 (clone 145-2C11; American Type Culture Collection, Manassas, VA) and 2 μg of anti-CD28 (clone 37.51; BD PharMingen, San Diego, CA) i.v. Six hours following injection, T cells were purified from isolated splenocytes by depletion of B cells, macrophages, and class II-positive cells. In some experiments, purified T cells were further separated into CD45RB high cells (naive) and CD45RB low cells (previously activated) with anti-CD45RB (clone 16A; BD PharMingen) and anti-rat magnetic beads from Qiagen (Valencia, CA). In vitro stimulation of T cells was performed using 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28. Poly(A) RNA purification and qPCR assays were performed, as above. IL-2 qPCR primers and probe were obtained from Applied Biosystems. Data between groups were analyzed for significance using an unpaired *t* test.

Results

Refinement of the proximal boundary of the Idd5.1 *interval*

Idd5.1 was initially defined using a panel of chromosome 1 congenic strains, including R8, R2, R467, and R444 (Fig. 1), as a 5.1-cM B10-derived resistance interval containing the candidate genes *Casp8*, *Cflar* (FLIP), *Cd28*, and *Ctla4* (1). We now report the development of the R974 strain (Fig. 1), which exhibits the same level of protection from T1D as the R444 strain ($p = 0.15$; Fig. 2*A*). Thus, the R974 and R444 strains have resistance alleles at both *Idd5.1* and *Idd5.2*. Delineation of the centromeric recombination point in R974 required the harvesting of additional NOD/B10 polymorphic microsatellites between and flanking the *Cd28* and *Ctla4* genes (Fig. 3). This search was greatly facilitated by the availability of both B6 and NOD genomic sequence in this region (see below). The centromeric boundary of *Idd5.1* is now defined as between *D1Mit249* and *Cd28distal3* (Fig. 3), thus eliminating *Cd28*, as well as *Casp8* and *Cflar* (FLIP) (Fig. 1) as candidates for *Idd5.1*. These findings are consistent with those of Lamhamedi-Cherradi et al. (2), in which *Casp8* and *Cflar* were excluded from the *Idd5.1* region. Importantly, Lamhamedi-Cherradi et al. based their *Idd5.1* mapping on NOD.B6 congenic strains rather than NOD.B10 congenic strains as used in the current study; thus, the similar findings strongly suggest that the highly related B6 and B10 strains share a T1D resistance allele at *Idd5.1*. This has important implications because the major public sequencing effort of the mouse genome has made use of B6 DNA.

Refinement of the distal boundary of the Idd5.1 *interval: discovery of a cold spot of recombination within* Idd5.1

The distal boundary of *Idd5.1* was first defined by the R2 strain (Fig. 1), which has a recombination breakpoint between *D1Mit303* and *D1Mit300* (1). We now report the development of a recombinant congenic strain, R46, which is protected from T1D as compared with the NOD strain ($p = 0.00006$), but not to the extent seen in R444 ($p = 0.0002$, R46 vs R444) (Fig. 2). Consistent with the protection seen previously with the R467 recombinant strain (Fig. 1) that was shown to have a resistant allele at *Idd5.1*, but not at *Idd5.2* (1), the R46 recombinant strain has the degree of protection conferred by the *Idd5.1* region alone (Fig. 2). The R46 strain defines the distal boundary of *Idd5.1* within the *Nrp2* (neuropilin-2) gene (Fig. 3) showing that the *Idd5.1* locus is up to 2.1 Mb in length. The R426 and R193 strains demonstrated the same protection from disease as R46 ($p = 0.61$ and $p = 0.13$ vs R46, respectively), consistent with the presence of only the B10 allele of *Idd5.1* in both of these new recombinant strains.

As the recombination breakpoints near the *Idd5.1* boundaries were analyzed, we found that three recombination events had occurred in 2194 meioses between markers *D1Mit249* (~76 kb centromeric of *Ctla4*) and *D1Mit303* (Fig. 3). Because *D1Mit303* is ~2 Mb distal of *D1Mit249*, 22 recombinant mice would have been expected rather than the three observed. In addition, of the six mice with samples available for testing (of nine recombinants total) that had recombined between *D1Mit303* and *D1Mit300* (a distance of ~1 Mb), all six recombined in a 280-kb region between *AL645534GA* and *D1Mit300*. This nonlinear recombination is not atypical (9), but does present practical difficulties when it is desirable to construct a congenic strain having a specific recombination point that is relatively close to, but not at, the hot spot of recombination.

Gene content of the Idd5.1 *region: assessment of functional candidacy*

Analysis of the *Idd5.1* gene content from the B6 BAC-based genomic sequence (Fig. 3) indicates that the 2.1-Mb region contains only four genes, *Ctla4*, *Icos*, *Als2cr19*, and *Nrp2*. Whereas *Ctla4* and *Icos* are clearly strong functional candidates for *Idd5.1* due to their important functions in the immune system (10, 11), *Als2cr19* (12) and *Nrp2* (13) are less likely candidates for influencing autoimmune diabetes because they have no recognized function in either β cell development or the immune system. Human NRP2 is a receptor for both semaphorins, which regulate neuronal guidance, and vascular endothelial growth factor, an angiogenic molecule. Disruption of the mouse NRP2 gene impairs yolk sac development and embryonic angiogenesis. It is noted, however, that NRP1 has a central role in T cell activation (14), and it is possible that NRP2 has a yet undiscovered role in the immune system. *Als2cr19* is a partitioning defective-like protein. Partitioning defective proteins were first discovered in *Caenorhabditis elegans* and are essential for asymmetric cell division and polarized growth. *Als2cr19* has 23 exons extending over 1 Mb and *Nrp2* has 17 exons over 112 kb. The R46 distal recombination point is between exons 7 and 17 of *Nrp2*. Thus, the small number of genes in the *Idd5.1* region is accounted for, in part, by the existence of one, very large gene (*Als2cr19*) extending over nearly one-half of the 2.1-Mb interval. Finally, from the currently known functions of the four genes in the interval, and results from genetic analysis of their

Table II. *Protein alignment of* Icos *and putative soluble splice variant*[a]

ICOS	MKPYFCRVFVFCFLIRLLTGEINGSADHRMFSFHNGGVQISCKY
Soluble ICOS	MKPYFCRVFVFCFLIRLLTGEINGSADHRMFSFHNGGVQISCKY
ICOS	PETVQQLKMRLFREREVLCELTKTKGSGNAVSIKNPMLCLYHL
Soluble ICOS	PETVQQLKMRLFREREVLCELTKTKGSGNSVSIKNPMLCLYHL
ICOS	SNNSVSFFLNNPDSSQGSYYFCSLSIFDPPPFQERNLSGGYLHIY
Soluble ICOS	SNNSVSFFLNNPDSSQGSYYFCSLSIFDPPPFQERNLSGGYLHIY
ICOS	ESQLCCQLKL**WLPVGCAAFVVVLLFGCILIIWF**SKKKYGSSVH
Soluble ICOS	----------*EIWIQ*CA
ICOS	DPNSEYMFMAAVNTNKKSRLAGVTS

Bold = transmembrane domain, underline = signal peptide, italics = different OFR.

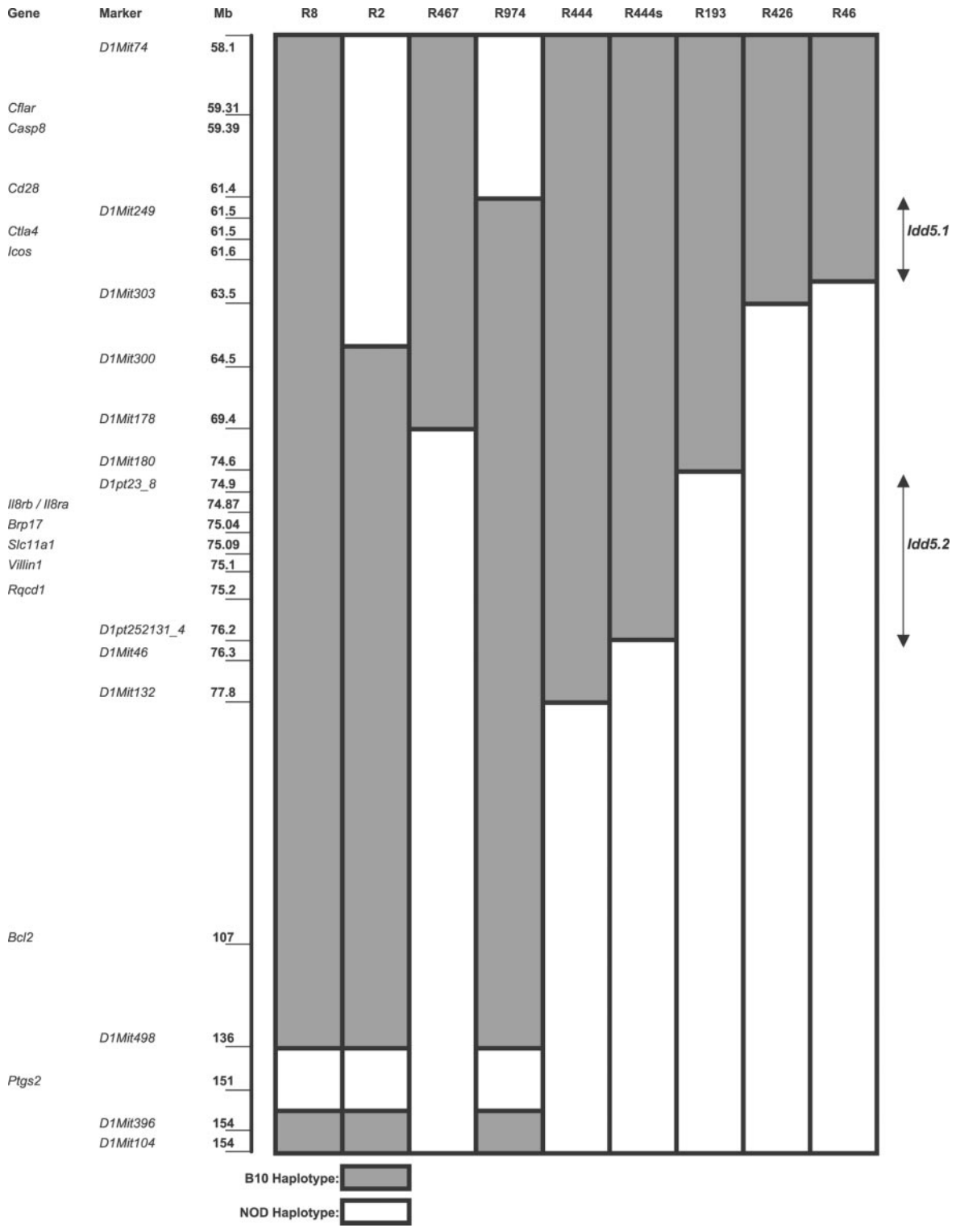

FIGURE 1. Description of genetic intervals present in the *Idd5* congenic strains. Regions filled and open represent the B10 and NOD haplotypes, respectively. The *Idd5.1* and *Idd5.2* regions are designated by vertical arrows.

orthologues in human T1D (6), *Ctla4* and *Icos* remain the favored candidates by this functional criterion.

B6-NOD sequence comparison of genes in the Idd5.1 *region*

We have obtained NOD-derived BAC-based sequence from portions of the *Idd5.1* region, including the interval containing *Ctla4* and *Icos* as well as one exon and portions of two introns of *Als2cr19* (Fig. 3). Table III details the polymorphisms seen in the regions in which sequences can be directly compared. Numerous SNPs are observed in intronic and 3′ and 5′ regulatory regions of *Ctla4* and *Icos* and indicate that both genes are within a region that is ancestrally distinct between NOD and B6 mice (15, 16). Although many SNPs in these regions were observed, no variants were present in either gene within any of the 5′ or 3′ splice sites or branch points. There are two exonic SNPs, one each in *Ctla4* and *Icos*. The exonic SNP present in *Icos* causes a nonconservative

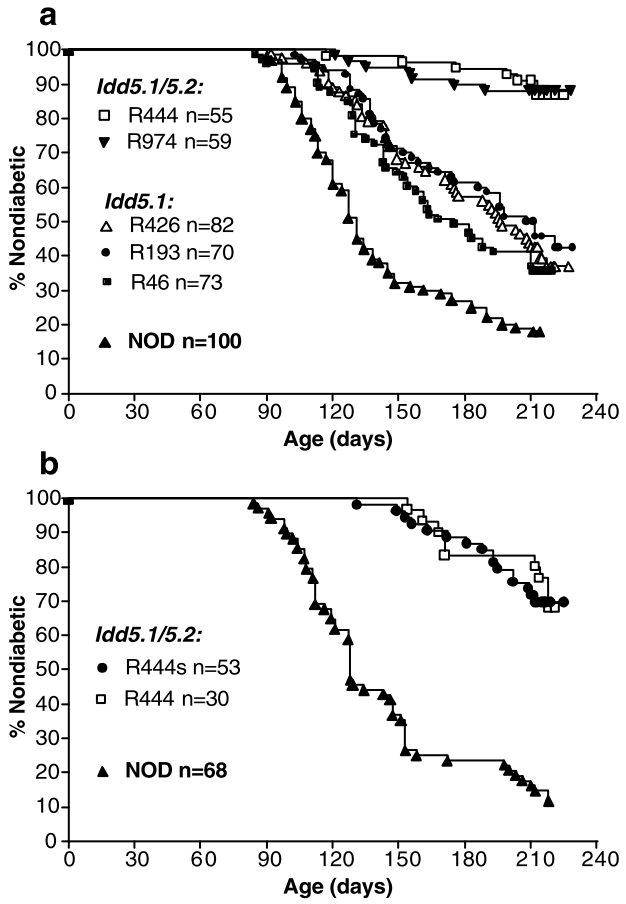

FIGURE 2. Frequency of diabetes in female *Idd5* congenic mice.

amino acid change at amino acid residue 7, where the arginine in the B6 ICOS protein is replaced by a histidine in the NOD molecule. Although residue 7 is part of the putative leader sequence of the ICOS protein and should, therefore, be removed before expression, it is possible that the amino acid sequence change could alter expression levels at the cell surface (17).

The one exon of *Als2cr19* that can be compared between B6 and NOD, exon 2, has an identical nucleotide sequence (Table III). There is also very little sequence difference in the intronic sequence surrounding exon 2 (0.3 SNP/10 kb). This could be due to the fact that these two strains are identical by descent (15, 16) in this particular region as compared with the nearby interval containing *Ctla4* and *Icos*, where 12–52 SNPs/10 kb were observed (Table III). This possibility is substantiated by the observation that of 21 microsatellites examined in silico in the *Als2cr19* region, in which there is sequence coverage from both B6 and NOD BACs, none was polymorphic (Fig. 3). A similar observation was made distal to this region in which only 1 (*AL662815TA*) of 10 microsatellites genotyped were polymorphic and *AL662815TA* itself differed by only 2 nt between NOD and B6.

Ctla4 *and* Icos *splice variants*

Because the vast majority of *Ctla4* and *Icos* SNPs are in introns and putative regulatory regions, we tested for steady state mRNA expression differences in T cells from NOD and NOD.B10 *Idd5.1* congenic strains of mice. mRNAs encoding full-length ICOS and CTLA-4 and sCTLA-4, a form of CTLA-4 observed by many groups, but whose physiologic function is poorly understood (6), were examined (18, 19). In addition, we also searched for novel splice forms of *Ctla4* and *Icos* in normal and activated T cells. Although the *Ctla4* SNP in exon 2 does not cause an amino acid change, we have recently reported that this silent B6/NOD SNP at residue 77 in exon 2, the exon encoding the ligand binding domain of CTLA-4, is a critical nucleotide in the creation of a novel splice variant (6). This isoform is referred to as liCTLA-4 (6). The variation at residue 77 results in a 4-fold higher expression of liCTLA-4 mRNA in B10 *Idd5.1* T cells as compared with NOD T cells (6) (also see Figs. 4, 5, 6, and 7). The B6/NOD SNP in exon 2 of *Ctla4* alters the sequence of a putative exonic splicing silencer motif, thereby changing the efficiency of producing mRNA for liCTLA-4, but not affecting the production of flCTLA-4 or sCTLA-4 (6).

Potential splice variants of ICOS were assessed by RT-PCR, as described in *Materials and Methods*. Unlike CTLA-4, in which four mRNA species are present (6), only a single splice variant encoding a putative soluble form of ICOS, sICOS, could be documented (see *Materials and Methods*). In addition, no reproducible, genotype-related expression differences of either full-length ICOS (flICOS) or sICOS mRNA have been observed in unstimulated or activated T cells (Fig. 4). However, as shown in the accompanying study (20), genotype-related expression differences in ICOS protein on the cell surface of T cells have been observed.

Increased expression of mRNA encoding all three CTLA-4 isoforms in CD45RB low T cells

Because a genotype-dependent production of liCTLA-4 was observed previously, it is important to determine whether this is due to differential expression depending on the maturation state of the cells examined. It has been demonstrated previously that flCTLA-4 expression at the protein level is lower in CD45RB high (naive) compared with CD45RB low (memory) T cells (21, 22). We, therefore, isolated CD45RB high and CD45RB low T cells from NOD and R444 spleens and tested them for expression of flCTLA-4, liCTLA-4, and sCTLA-4 (Fig. 5). For all three isoforms, expression was 16- to 64-fold (4–6 qPCR cycles) higher in memory CD45RB low $CD4^+$ T cells as compared with naive CD45RB high $CD4^+$ T cells. A similar, albeit smaller (2–4 qPCR cycles more in memory cells), difference was observed for ICOS and sICOS mRNA (data not shown). Additionally, the liCTLA-4 was differentially expressed in both subsets of cells, although the quantitative detection of liCTLA-4 mRNA was problematic in naive NOD T cells due to its very low abundance in this cell type.

Genetic control of liCTLA-4 expression

Strains of mice having either the NOD or B6 allele at *Ctla4* were assessed for liCTLA-4 mRNA expression. Spleen cells were tested following in vivo activation with anti-CD3/CD28. To account for individual differences in the numbers of CD45RB low memory T cells, the cell subset in which the majority of CTLA-4 expression is found (Fig. 5), the liCLTA-4 mRNA level in each mouse was normalized against its own flCTLA-4 mRNA level. Results from congenic strains recombining near the CTLA-4 locus were consistent with the hypothesis that an altered splicing preference is determined by the exon 2 SNP: R974 mice (B10 allele at *Idd5.1*) had high levels of liCTLA-4 mRNA, whereas R2 mice (NOD allele at *Idd5.1*) had low levels (Fig. 6). Finally, we tested several other congenic strains not having the *Idd5.1* B10 allele, including some having combinations of *Idd* loci that confer profound protection from T1D such as congenic strains with disease-resistant alleles at *Idd3/10/18* and *Idd9.1/9.2/9.3* (5), and observed that they produced low levels of liCTLA-4 mRNA (Fig. 6). In contrast, when B10 *Idd5.1* is present together with the B10 *Idd5.2* and B6 *Idd3* loci in *Idd3/5* mice, a strain in which only 1% of the mice develop T1D and <10% develop more than minimal insulitis or

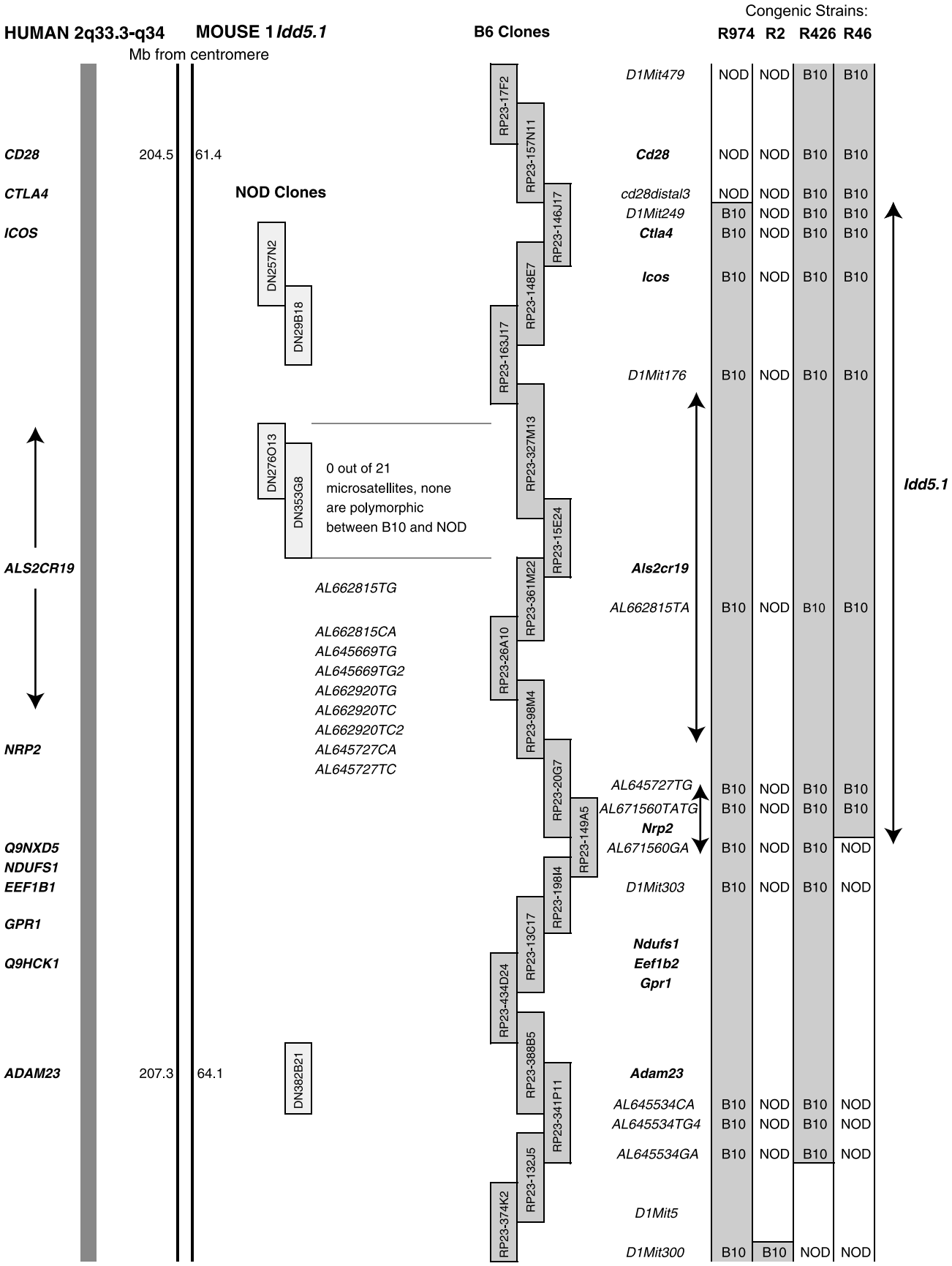

FIGURE 3. Fine mapping of *Idd5.1*. The proximal and distal boundaries defined by the R974 and R46 strains, respectively, define the four genes encoded within the *Idd5.1* interval: *Ctla4*, *Icos*, *Alscr19*, and *Nrp2*. These genes are also represented in the human orthologous region 2q33.3-q34. Dark gray clones with the prefix RP23 are B6 clones and all have complete sequence. Light gray clones with the prefix DN are NOD clones and all have complete sequence. Microsatellite markers that are not polymorphic between NOD and B10 are shown to the *left* of the B6 contig.

Table III. *Genetic variation between NOD and B6 in the Idd5.1 region*[a]

	Size in B6 (bp)	SNPs	Coding Changes	in/dels	SNPs/kb
Region proximal to *Ctla4*	37,208	194		81	5.2
Ctla4					
5′ UTR[b]	1,049	1		0 }	
Exon 1	109	0	0	0 }	
Intron 1	3,140	5		2 }	
Exon 2	348	1	0	0 }	
Intron 2	448	0		0 }	1.3
Exon 3	110	0	0	0 }	
Intron 3	1,266	2		1 }	
Exon 4	105	0	0	0 }	
3′ UTR	4,050	5		1 }	
Region between *Ctla4* and *Icos*	57,810	153		47	2.6
Icos					
5′ UTR	1,371	4		3 }	
Exon 1	58	1	1	0 }	
Intron 1	15,683	37		13 }	
Exon 2	339	0	0	0 }	
Intron 2	599	1		1 }	
Exon 3	107	0	0	0 }	1.9
Intron 3	804	2		1 }	
Exon 4	84	0	0	0 }	
Intron 4	2,117	2		5 }	
Exon 5	14	0	0	0 }	
3′ UTR	3,517	1		1 }	
Region distal to *Icos*	10,680	13		3	1.2
Als2cr19 intron 1	60,350	1		3 }	
Als2cr19 exon 2	102	0	0	0 }	0.03
Als2cr19 intron 2	126,716	5		6 }	

[a] The number of SNPs and in/dels between NOD and B6 is shown for the portions of the *Idd5.1* interval having complete sequence from both strains. Of the in/dels proximal to *Ctla4*, one is an L1 repeat (7400 bp) and one is a mouse endogenous retroviral sequence (6441 bp); both are only present in NOD. Of the in/dels distal to *Ctla4*, one is an L1 repeat (7025 bp), also only present in NOD.

[b] UTR, untranslated region.

anti-insulin autoantibodies (5), the higher level of liCTLA-4 mRNA expected from the B10 allele at *Idd5.1* was observed (Fig. 6). B6 mice congenic for the NOD *Idd5.1* allele produced low levels of liCTLA-4 mRNA compared with B6 or B10 mice having their normal *Idd5.1* B6 or B10 allele (Fig. 6), an expected result if differential expression of liCTLA-4 is due to a *cis*-DNA variation intrinsic to the allele. The activated splenocytes described in Fig. 6 were also examined for sCTLA-4, ICOS, and sICOS mRNA levels by qPCR, and no genotype-related expression differences were observed (data not shown).

Expression of liCTLA-4 in an in vivo Ag-specific response

To examine the expression of liCTLA-4 mRNA under more physiological conditions, NOD and R444 mice were primed with SRBC and later challenged in the footpad with SRBC. Twenty-four hours after challenge, mRNA extracted from the footpads demonstrated a reproducible differential level of expression between NOD and R444 of liCTLA-4, but not of flCTLA-4, sCTLA-4, flICOS, IFN-γ, IL-2, and IL-10 (Fig. 7 and data not shown). By qPCR, sICOS mRNA was not detected in these samples. There was no difference in the degree of the delayed-type hypersensitivity response as measured by footpad swelling between the two groups of mice in either experiment (data not shown).

Refinement of the proximal and distal boundaries of the Idd5.2 *interval*

Idd5.2 was previously defined to be in a 5.1-cM region distal to *Idd5.1*, and is, in combination with *Idd5.1*, critical for the full protection mediated by the *Idd5* region (1). New congenic strains were developed to further define the *Idd5.2* interval: R444s, R193, and R426 (Figs. 1, 2, and 8). The frequency of diabetes does not differ between R444 and R444s (Fig. 2*B*, $p = 0.66$). However, R193 is less protected from disease than R444 (Fig. 2*A*, $p = 0.001$), indicating that R193 has lost the protective allele at *Idd5.2*. Supporting this hypothesis is the observation that disease protection in R193 is indistinguishable from R426 ($p = 0.92$) and R46 ($p = 0.56$). All three strains, R193, R426, and R46, are protected as compared with the NOD parental strain ($p < 0.0006$ for each comparison), and all, therefore, have the *Idd5.1* allele. The level of disease protection observed in the R193, R426, and R46 congenic strains is very similar to that reported for the R1, R39, and R67 *Idd5.1* NOD.B6 strains (2), consistent with B6 and B10 sharing a T1D-resistant allele in *Idd5.1*.

Interrogation of the *Idd5.2* region between the proximal and distal boundaries as defined by the R193 and R444s strains (Figs. 1 and 8) was performed using the B6 sequence available from mouse EnsEMBL. In stark contrast to the gene-poor *Idd5.1* region detailed above, an extremely gene-rich *Idd5.2* region was found: at least 45 genes are present within the 1.52-Mb interval (Fig. 8). *Nramp1*, known to be functionally polymorphic between B6/B10 (expressing a nonfunctional allotype) and NOD (expresses the functional protein), remained within the *Idd5.2* interval.

The gene content in Idd5.1 *and* Idd5.2 *is conserved with human 2q33.3-q34 and 2q35, respectively*

A comparative analysis of draft sequence from the B6 BAC tile path with the orthologous human sequence revealed 4 and 45 orthologous genes within the 2.1-Mb *Idd5.1* and 1.52-Mb *Idd5.2* intervals, respectively (Figs. 3 and 8). The 4 genes in *Idd5.1* were examined for the extent of identity at the amino acid level between the mouse and human sequences. As has been described previously, amino acid identity for CTLA-4 and ICOS with their human counterparts is 76 and 72% (23, 24), respectively, whereas the

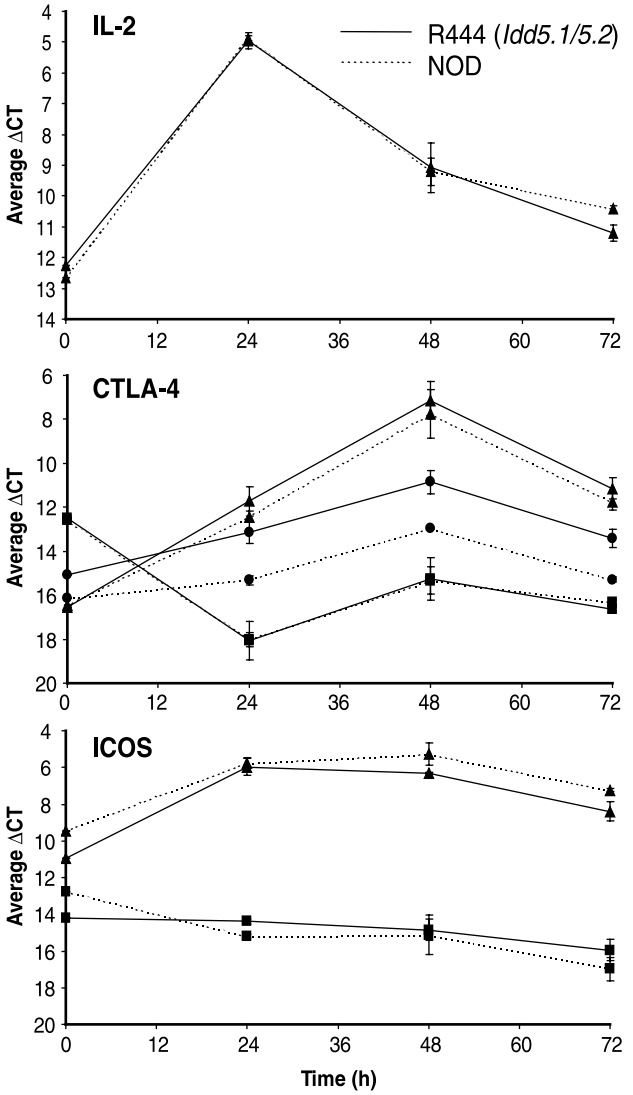

FIGURE 4. Correlation of genotype with levels of liCTLA-4 mRNA. Purified T cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in vitro and then assessed at various times for mRNA levels by reverse transcription and qPCR (TaqMan). Values are normalized to β_2-microglobulin mRNA with ΔCT, representing the difference in CT (cycle threshold) values between the experimental and control genes. Error bars represent the SD of the mean of duplicate samples. The dashed and solid lines represent NOD and R444 (*Idd5.1/5.2*) T cells, respectively. In the case of CTLA-4 and ICOS, triangles and squares represent the full-length and soluble isoforms, respectively. Circles represent the liCTLA-4 isoform.

ALS2CR19 and *NRP2* genes have 85 and 94% identity with the equivalent human genes, respectively.

Discussion

The challenge of defining genes underlying complex genetic traits, including those causing disease, remains formidable. In defining genes in a model of disease, such as the NOD mouse that develops T1D, key tools include the development of congenic strains that are protected from disease because of a genetic region derived from a disease-resistant strain. Ultimately, the best that can be obtained from such strains is the narrowing of a region containing the disease gene to 0.5–3 Mb, depending on the frequency of recombination in the region under study. At this point, a systematic gene identification strategy is to obtain a complete sequence of the defined region, determine the gene content, and, from comparative

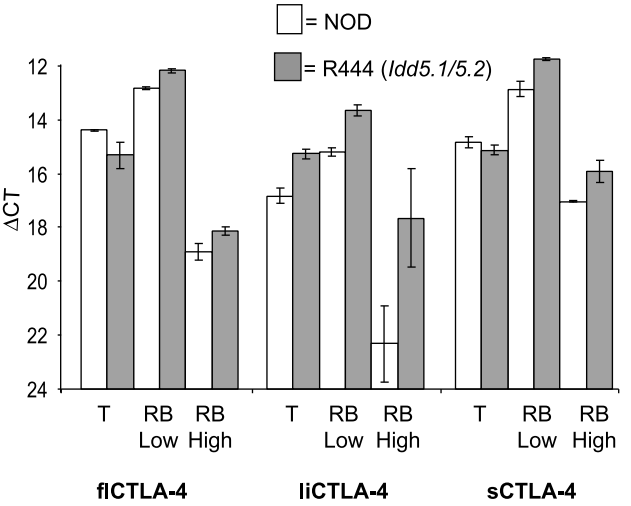

FIGURE 5. CD45RB low cells are enriched for flCTLA-4, liCTLA-4, and sCTLA-4 mRNA expression. ΔCT (cycle threshold) values were determined as detailed in Fig. 4.

sequencing of the same region in the other parental strain, identify all SNPs. Unfortunately, these steps remain time consuming and expensive and only represent the beginning of the search for the causative SNP in a polygenic disease. It will be rare for a susceptibility locus to be identified from sequence information alone; one case would be if the susceptible and resistant strains are identical by descent in the defined genetic interval and there is a functional de novo mutation that has occurred in one of the two strains being compared. Unfortunately, as is the case for *Idd5.1* and *Idd5.2*, most genes that influence a complex disease will be one of many linked genes within a distinct ancestral haplotype (15, 16) that differs between the susceptible and resistant parental strains. Therefore, many DNA variants are present (>20 SNPs per 10 kb), making the identification of the causal SNP or SNPs much more difficult.

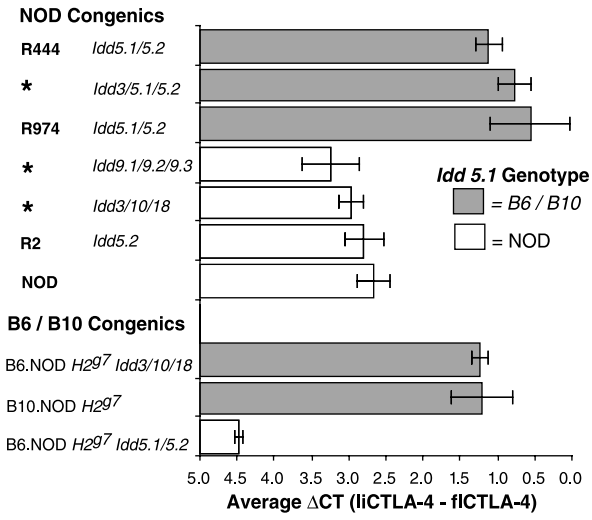

FIGURE 6. The *Idd5.1* region controls expression levels of liCTLA-4 in congenic strains. ΔCT (cycle threshold) values for flCTLA-4 and liCTLA-4 were determined as in Fig. 4. The levels of liCTLA-4 were normalized, as described in the text, by subtracting the flCTLA-4 ΔCT from the liCTLA-4 ΔCT. The number of mice tested per group ranged from 3 to 10. The mean and SE for each group of mice are shown. *, These strains are described in detail in Robles et al. (5). The B6.NOD congenic strains were developed by Wakeland and colleagues (27). The B10.$H2^{g7}$ strain has been described previously (28).

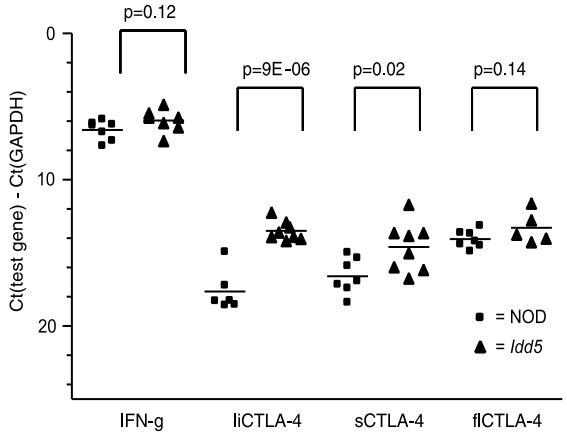

FIGURE 7. The *Idd5.1* region controls expression levels of liCTLA-4 in vivo. Expression levels of mRNA for IFN γ, flCTLA-4, liCTLA-4, sCTLA-4, and flICOS were determined as in Fig. 4. Mice were immunized i.v. with SRBC and challenged in one footpad with SRBC. The delayed-type hypersensitivity response to SRBC peeked at 24 h postchallenge, the time at which mRNA from the footpads of the challenged mice was harvested.

The disease-causative *Idd* intervals described in this study, *Idd5.1* and *Idd5.2*, represent two contrasting consequences of using the gene identification strategy outlined above. The larger 2.1-Mb *Idd5.1* region contains only four genes, two of which are strong functional candidates, whereas the physically smaller 1.65-Mb *Idd5.2* region contains 45 genes. Because both of these regions have already been delineated using thousands of meiotic events to select specific recombinant mice, it is clear that even at the end of the congenic strain development phase, the investigator is left with regions containing many genes containing hundreds or thousands of SNPs, nearly all of which will be irrelevant for the disease phenotype, even though some of the variants may have functional effects. Although we propose that *Idd5.1* is *Ctla4* and the molecular basis of the phenotypic change is caused by the exon 2 SNP that determines the production of liCTLA-4, it remains possible that a SNP (or SNPs) in the ICOS gene is responsible for the disease phenotype, or that variants at *Ctla4* and *Icos* together are causative. Although no genotype-dependent expression or splicing difference was noted for ICOS mRNA under the conditions tested in this study, it may well be that under different stimulation conditions or if expression is assessed in specific cell types (see Ref. 20), ICOS will be differentially expressed in NOD vs *Idd5.1* mice due to one or more SNPs in *Icos*. It is also possible that the SNP present in the leader sequence of the ICOS molecule could cause a change in protein expression that would not be associated with an alteration in the steady state level of *Icos* transcription. An additional complexity of the *Idd5.1* locus is that a difference in ICOS expression could be a downstream consequence of a genetic variant in *Ctla4*. Riley et al. (25) found that CTLA-4 engagement reduced expression of ICOS on the cell surface. Thus, the higher expression of liCTLA-4 in activated T cells having the

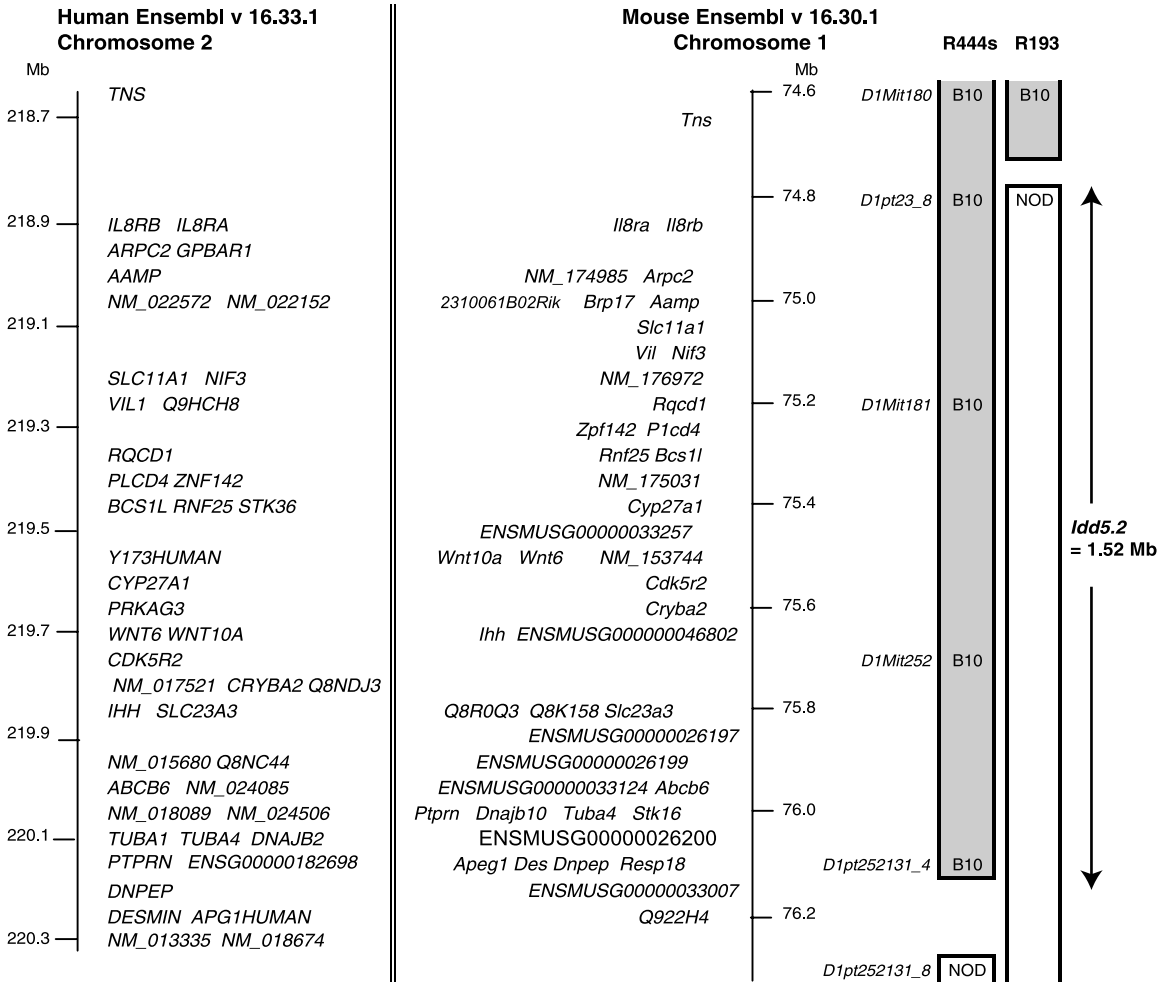

FIGURE 8. Fine mapping of the *Idd5.2* interval. Genes present in the orthologous human genomic region are also indicated.

B10 allele at *Idd5.1* would increase negative signaling, thereby decreasing the up-regulation of ICOS on the cell surface. This hypothesis could explain some of the observations reported in the accompanying study by Greve et al. (20), in which higher cell surface expression of ICOS on activated T cells is correlated with the NOD haplotype at *Idd5.1*, a genotype that confers lower liCTLA-4 expression.

To prove that a variant gene within a disease-modifying genetic interval is the disease-causing allele requires that the SNP change the course of the disease. This is true even for a gene with alleles having a compelling functional difference. To determine whether the altered splicing preference of CTLA-4 is in fact ultimately responsible for changing disease susceptibility, we are developing an *Idd5.1* congenic mouse strain in which the donor segment has been derived from an inbred strain in which a historical recombination event has occurred between *Ctla4* and *Icos*, thereby breaking apart the extended haplotypes present in B10 and NOD mice. If data from this novel congenic strain support a causative role for residue 77, the next experiment would be to knockin the B10 exon 2 *Ctla4* SNP into the NOD genome to determine whether a single SNP is sufficient to not only alter liCTLA-4 expression, but also ICOS expression and disease frequency. To this end, we have developed (NOD × 129) embryonic stem cells to target the NOD genome (26).

Another argument for the likelihood that *Ctla4* rather than *Icos* functions as *Idd5.1* is the observation that the *Ctla4* knockout mouse displays a much more severe phenotype than the *Icos* knockout mouse (10, 11). This suggests that quantitative variation of *Ctla4* would be more likely to confer a selective pressure than quantitative variation of *Icos*. The association of human autoimmune diseases with *CTLA4*, but not *ICOS*, is consistent with this hypothesis (6).

References

1. Hill, N. J., P. A. Lyons, N. Armitage, J. A. Todd, L. S. Wicker, and L. B. Peterson. 2000. The NOD *Idd5* locus controls insulitis and diabetes and overlaps the orthologous CTLA4/IDDM12 and NRAMP1 loci in humans. *Diabetes 49:1744*.
2. Lamhamedi-Cherradi, S. E., O. Boulard, C. Gonzalez, N. Kassis, D. Damotte, L. Eloy, G. Fluteau, M. Levi-Strauss, and H. J. Garchon. 2001. Further mapping of the Idd5.1 locus for autoimmune diabetes in NOD mice. *Diabetes 50:2874*.
3. Vidal, S., P. Gros, and E. Skamene. 1995. Natural resistance to infection with intracellular parasites: molecular genetics identifies Nramp1 as the Bcg/Ity/Lsh locus. *J. Leukocyte Biol. 58:382*.
4. Jabado, N., P. Cuellar-Mata, S. Grinstein, and P. Gros. 2003. Iron chelators modulate the fusogenic properties of *Salmonella*-containing phagosomes. *Proc. Natl. Acad. Sci. USA 100:6127*.
5. Robles, D. T., G. S. Eisenbarth, N. J. Dailey, L. B. Peterson, and L. S. Wicker. 2003. Insulin autoantibodies are associated with islet inflammation but not always related to diabetes progression in NOD congenic mice. *Diabetes 52:882*.
6. Ueda, H., J. M. Howson, L. Esposito, J. Heward, H. Snook, G. Chamberlain, D. B. Rainbow, K. M. Hunter, A. N. Smith, G. Di Genova, et al. 2003. Association of the T-cell regulatory gene *CTLA4* with susceptibility to autoimmune disease. *Nature 423:506*.
7. Lyons, P. A., and L. S. Wicker. 1999. Localizing quantitative trait loci in the NOD mouse model of type 1 diabetes. In *Genes and Genetics of Autoimmunity*, Vol. 1. A. N. Theofilopoulos, ed. Karger, Basel, p. 208.
8. Overbergh, L., D. Valckx, M. Waer, and C. Mathieu. 1999. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine 11:305*.
9. Petes, T. D. 2001. Meiotic recombination hot spots and cold spots. *Nat. Rev. Genet. 2:360*.
10. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity 3:541*.
11. McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, and A. H. Sharpe. 2001. ICOS is critical for CD40-mediated antibody class switching. *Nature 409:102*.
12. Nagai-Tamai, Y., K. Mizuno, T. Hirose, A. Suzuki, and S. Ohno. 2002. Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells. *Genes Cells 7:1161*.
13. Takashima, S., M. Kitakaze, M. Asakura, H. Asanuma, S. Sanada, F. Tashiro, H. Niwa, J. Miyazaki Ji, S. Hirota, Y. Kitamura, et al. 2002. Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc. Natl. Acad. Sci. USA 99:3657*.
14. Tordjman, R., Y. Lepelletier, V. Lemarchandel, M. Cambot, P. Gaulard, O. Hermine, and P. H. Romeo. 2002. A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. *Nat. Immunol. 3:477*.
15. Wade, C. M., E. J. Kulbokas III, A. W. Kirby, M. C. Zody, J. C. Mullikin, E. S. Lander, K. Lindblad-Toh, and M. J. Daly. 2002. The mosaic structure of variation in the laboratory mouse genome. *Nature 420:574*.
16. Wiltshire, T., M. T. Pletcher, S. Batalov, S. W. Barnes, L. M. Tarantino, M. P. Cooke, H. Wu, K. Smylie, A. Santrosyan, N. G. Copeland, et al. 2003. Genome-wide single-nucleotide polymorphism analysis defines haplotype patterns in mouse. *Proc. Natl. Acad. Sci. USA 100:3380*.
17. Van der Linde, K., P. P. Boor, L. A. Sandkuijl, M. A. Meijssen, H. F. Savelkoul, J. H. Wilson, and F. W. de Rooij. 2003. A Gly15Arg mutation in the interleukin-10 gene reduces secretion of interleukin-10 in Crohn disease. *Scand. J. Gastroenterol. 38:611*.
18. Magistrelli, G., P. Jeannin, N. Herbault, A. Benoit De Coignac, J. F. Gauchat, J. Y. Bonnefoy, and Y. Delneste. 1999. A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. *Eur. J. Immunol. 29:3596*.
19. Oaks, M. K., and K. M. Hallett. 2000. Cutting edge: a soluble form of CTLA-4 in patients with autoimmune thyroid disease. *J. Immunol. 164:5015*.
20. Greve, B., L. Vijayakrishnan, A. Kubal, R. A. Sobel, L. B. Peterson, L. S. Wicker, and V. K. Kuchroo. 2004. The diabetes susceptibility locus *Idd5.1* on mouse chromosome 1 regulates ICOS expression and modulates murine experimental autoimmune encephalomyelitis. *J. Immunol. 173:0000*.
21. Birkeland, M. L., P. Johnson, I. S. Trowbridge, and E. Pure. 1989. Changes in CD45 isoform expression accompany antigen-induced murine T-cell activation. *Proc. Natl. Acad. Sci. USA 86:6734*.
22. Lee, W. T., X. M. Yin, and E. S. Vitetta. 1990. Functional and ontogenetic analysis of murine $CD45R^{hi}$ and $CD45R^{lo}$ $CD4^+$ T cells. *J. Immunol. 144:3288*.
23. Dariavach, P., M. G. Mattei, P. Golstein, and M. P. Lefranc. 1988. Human Ig superfamily CTLA-4 gene: chromosomal localization and identity of protein sequence between murine and human CTLA-4 cytoplasmic domains. *Eur. J. Immunol. 18:1901*.
24. Mages, H. W., A. Hutloff, C. Heuck, K. Buchner, H. Himmelbauer, F. Oliveri, and R. A. Kroczek. 2000. Molecular cloning and characterization of murine ICOS and identification of B7h as ICOS ligand. *Eur. J. Immunol. 30:1040*.
25. Riley, J. L., P. J. Blair, J. T. Musser, R. Abe, K. Tezuka, T. Tsuji, and C. H. June. 2001. ICOS costimulation requires IL-2 and can be prevented by CTLA-4 engagement. *J. Immunol. 166:4943*.
26. Brook, F. A., E. P. Evans, C. J. Lord, P. A. Lyons, D. B. Rainbow, S. K. Howlett, L. S. Wicker, J. A. Todd, and R. L. Gardner. 2003. The derivation of highly germline-competent embryonic stem cells containing NOD-derived genome. *Diabetes 52:205*.
27. Yui, M. A., K. Muralidharan, B. Moreno-Altamirano, G. Perrin, K. Chestnut, and E. K. Wakeland. 1996. Production of congenic mouse strains carrying NOD-derived diabetogenic genetic intervals: an approach for the genetic dissection of complex traits. *Mamm. Genome 7:331*.
28. Lee, M. S., R. Mueller, L. S. Wicker, L. B. Peterson, and N. Sarvetnick. 1996. IL-10 is necessary and sufficient for autoimmune diabetes in conjunction with NOD MHC homozygosity. *J. Exp. Med. 183:2663*.