

Congenic Mapping of the Type 1 Diabetes Locus, *Idd3*, to a 780-kb Region of Mouse Chromosome 3: Identification of a Candidate Segment of Ancestral DNA by Haplotype Mapping

Paul A. Lyons,⁴ Nicola Armitage, Fabio Argentina, Paul Denny,¹ Natasha J. Hill, Christopher J. Lord, Mary Beth Wilusz,² Laurence B. Peterson,² Linda S. Wicker,³ and John A. Todd

Department of Medical Genetics, Wellcome Trust Centre for the Study of Molecular Mechanisms in Disease, University of Cambridge, Cambridge, CB2 2XY, UK; and the Departments of ³Immunology and Rheumatology and ²Pharmacology, Merck Research Laboratories, Rahway, New Jersey 07065, USA

Type 1 diabetes in the nonobese diabetic (NOD) mouse arises as a consequence of T cell-mediated destruction of the insulin-producing β cells of the pancreas. Although little is known of the events that initiate and subsequently drive β -cell destruction it is clear that the entire process is under complex genetic control. At present 19 loci have been mapped that influence the development of diabetes either at the level of initiation of insulinitis or at the level of progression from insulinitis to overt diabetes, or both. Previously, we have mapped one of these loci, *Idd3*, to a 0.35-cM interval on proximal mouse chromosome 3. In the present study we have narrowed the map position of this locus to an interval of 0.15 cM by a combination of novel congenic strains and an ancestral haplotype analysis approach. We have constructed a physical contig in bacterial artificial chromosome (BAC) clones across the minimal interval. Restriction mapping of the BAC contig placed the maximum size of the *Idd3* interval at 780 kb between the markers *D3Nds36* and *D3Nds76*. To refine further the *Idd3* interval we developed a series of novel single nucleotide polymorphisms (SNPs) and carried out haplotype analysis on DNA from mouse strains known to carry either *Idd3* susceptibility or protective alleles. This haplotype analysis identified a 145-kb segment of ancestral DNA between the microsatellite marker *D3Nds6* and the SNP *8l.3*. One haplotype of this ancestral segment of DNA is found in mouse strains carrying an *Idd3* susceptibility allele and another is found in mouse strains carrying an *Idd3* protective allele. Within the 780-kb congenically defined interval this 145-kb segment represents the most likely location for *Idd3*. The *Il2* gene, which encodes the cytokine interleukin 2 (IL2), maps to this interval and is a strong candidate for *Idd3*. To investigate whether sequence variation exists in the promoter region of the *Il2* gene, which might alter its expression, we sequenced the promoter region of the *Il2* gene from mouse strains carrying either an *Idd3* susceptibility or resistance allele. Two sequence variants were identified, neither of which fell in known regulatory elements within the *Il2* promoter. In agreement with this observation steady-state *Il2* mRNA levels showed no variation between susceptible and resistant mouse strains. These data suggest that the profound protection from diabetes seen in congenic mice carrying an *Idd3* protective allele is unlikely to be due to differences in the level of expression of the *Il2* gene. Instead, all of the current data support our hypothesis that *Idd3* corresponds to amino acid variation at the amino terminus of *Il2*.

[Sequence data reported in this paper have been deposited in GenBank and assigned the following accession numbers: AF19594, AF19595, and AF19596.]

Type 1 diabetes results from the autoimmune destruction of the insulin-producing β cells of the pancreas. A breakdown in immune homeostasis leads to a lymphocytic infiltration of the pancreatic islets of Langerhans, a process known as insulinitis, which in turn progresses

to β -cell destruction and overt disease. Although the molecular events that initiate and subsequently drive the process are uncertain it is clear that disease susceptibility is under complex genetic control.

The nonobese diabetic (NOD) mouse model of type 1 diabetes (Leiter 1998) has greatly facilitated the genetic dissection of type 1 diabetes. At present, 19 loci have been mapped that contribute to the development of type 1 diabetes in the NOD mouse (Leiter 1998;

¹Present address: MRC Mouse Genome Centre and Mammalian Genetics Unit, Harwell, Oxfordshire, OX11 0RD, UK.

⁴Corresponding author.

E-MAIL paul.lyons@cimr.cam.ac.uk; FAX +44 1223 762102.

Melanitou et al. 1998; Lyons and Wicker 1999). The approach we have adopted for the fine mapping of these insulin-dependent diabetes (*Idd*) susceptibility loci in the NOD mouse is one of congenic mapping (Wicker et al. 1994, 1995; Lord et al. 1995; Denny et al. 1997; Podolin et al. 1997).

Using this approach we have previously mapped the *Idd3* locus to a 0.35-cM interval on proximal mouse chromosome 3 between the microsatellite markers *D3Nds55* and *D3Nds40* (Denny et al. 1997). One gene known to map within this small interval is that encoding the cytokine interleukin-2 (IL2) (Denny et al. 1997). A growing body of evidence makes *Il2* a strong candidate for *Idd3*. A series of reports have shown that IL2 plays a central role in the development of self tolerance, with a lack of IL2 being associated with the development of autoimmune disease (Hunig and Schimpl 1998). It has been shown that IL2 is essential for activation-induced cell death of T cells mediated via the Fas pathway, a key mechanism of self-tolerance (Refaeli et al. 1998). We have shown previously that sequence polymorphisms exist between IL2 allotypes from different strains of mice (Ghosh et al. 1993; Denny et al. 1997) and that these polymorphisms, in exon 1 of *Il2*, segregate with susceptibility to diabetes (Denny et al. 1997). Moreover, one of the polymorphisms, the presence of proline rather than serine at position 6 of the mature IL2 protein, is associated with both the increased glycosylation of IL2 and diabetes susceptibility (Podolin et al. 2000). Despite the observed sequence differences between IL2 allotypes from diabetes-susceptible and -resistant strains no functional difference has been reported. Although the *Il2* promoter has been well characterized it is unknown whether variation exists in this region between mouse strains. Any variant that leads to an alteration in expression of *Il2* would be a strong candidate for the *Idd3* etiological mutation. Consistent with this possibility, reduced secretion of IL2 by mitogen-activated splenocytes has been reported previously in the NOD mouse (Serreze et al. 1989).

In this study we have refined the genetic mapping of *Idd3* to a 0.15-cM interval that still encompasses the *Il2* gene. We have constructed a physical contig of mouse BAC clones across the new minimal interval and by restriction mapping have determined that the maximum size of the *Idd3* interval is 780 kb. Haplotype analysis in mouse strains known to carry either an *Idd3* susceptibility or protective allele identifies the most likely location of *Idd3* as the 145-kb interval between the microsatellite marker *D3Nds6* and the single nucleotide polymorphism (SNP) 81.3. To identify potential regulatory polymorphisms we sequenced the promoter region of the *Il2* gene from mice carrying either susceptibility (NOD and 129) or protective (B6) alleles at *Idd3*. None of the identified variants within

the *Il2* promoter region fell in known regulatory elements. In agreement with this, no difference was observed in steady-state *Il2* mRNA levels, as assessed by semiquantitative RT-PCR, between mice carrying *Idd3* susceptibility or protective alleles.

RESULTS

Generation of New Variant Microsatellite Markers within the *Idd3* Interval

We have described previously the establishment of a YAC framework map across the *Idd3* region (Denny et al. 1997). To facilitate the isolation of additional polymorphic markers from within the *Idd3* region we identified mouse P1 and BAC clones positive for STSs developed from YACs spanning the *Idd3* interval. Mouse P1 and BAC libraries were screened by PCR with the following STSs: *B1R18STS*, *D3Nds36*, *D3Nds47*, *D3Nds6*, *D3Nds56*, *D3Nds51*, *D3Nds34*, *D3Nds46*, *D3Nds45*, and *D3Nds40*. Four new microsatellite markers, *D3Nds76*, *D3Nds77*, *D3Nds78*, and *D3Nds84*, were isolated from the clones mP284k17, mP315n15, mP305i10, and mP88b24, respectively. These new microsatellite markers were ordered with respect to *D3Nds55*, *D3Nds6*, *D3Nds34*, *D3Nds36*, and *D3Nds40* by genotyping the 944 progeny of an F₂ cross between NOD and the strain NOD.B6² (Lord et al. 1995). The following map order was obtained *D3Nds55*–(0.2 cM)–*D3Nds36*–(0.07 cM)–*D3Nds84*–(0 cM)–*D3Nds6*–(0 cM)–*D3Nds34*–(0.08 cM)–*D3Nds76*–(0 cM)–*D3Nds77*–(0 cM)–*D3Nds78*–(0 cM)–*D3Nds40* (Fig. 1). Where the marker order could not be resolved genetically the order was determined from the *Idd3* region physical map.

Narrowing of the *Idd3* Interval to 0.15 cM

Previously, we have mapped *Idd3* to a 0.35-cM interval between, but not including, the microsatellite markers

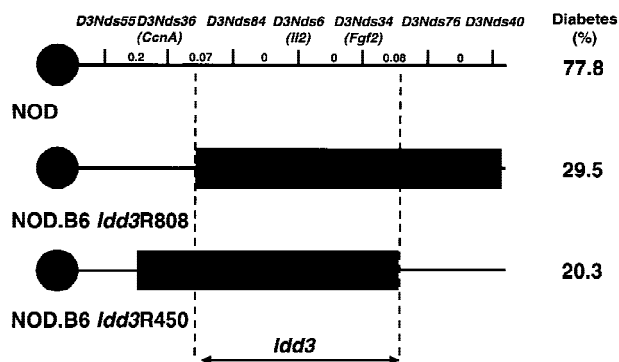


Figure 1 High-resolution genetic map of proximal chromosome 3 around *Idd3*. The lower bars show the genotypes for markers within the *Idd3* interval for the congenic strains NOD, B6 *Idd3*R450 and NOD, B6 *Idd3*R808. The solid bars indicate B6-derived genome and the numbers beside the bars indicate female diabetes frequencies at 7 months of age. Distances between markers are given in cM.

D3Nds55 and *D3Nds40* (Denny et al. 1997). To more precisely map the *Idd3* interval, the NOD.B6 *Idd3*R808 strain was developed from the previously described NOD.B6² congenic strain (Lord et al. 1995), a strain that carries the *Idd3* resistance allele. Genotyping the NOD. B6 *Idd3*R808 strain with polymorphic microsatellite markers that map to the *Idd3* interval showed that it was NOD derived at *D3Nds55* and *D3Nds36* and B6 derived at *D3Nds84*, *D3Nds6*, *D3Nds34*, *D3Nds76*, *D3Nds77*, *D3Nds78*, and *D3Nds40* (Fig. 1). This mapped the proximal boundary of its congenic segment to the 0.07-cM interval between *D3Nds36* and *D3Nds84*. The frequency of diabetes in females of this strain at 7 months is 29.5% (26/88) compared with 25.4% (17/67) in the NOD.B6² strain ($P > 0.05$) and 77.8% (63/81) in NOD ($P < 0.0001$). Thus, like NOD.B6², NOD. B6 *Idd3*R808 carries the *Idd3* resistance allele. Because NOD. B6 *Idd3*R808 carries the *Idd3* resistance allele the proximal boundary of *Idd3* must lie in the 0.07-cM interval between the markers *D3Nds36* and *D3Nds84* (Fig. 1).

The distal boundary of *Idd3* is defined by the congenic strains NOD.B6³ and NOD. B6 *Idd3*R450 (Lord et al. 1995). Typing these strains with the newly devel-

oped markers showed that both strains recombine in the 0.08-cM interval between *D3Nds34* and *D3Nds76* (Fig. 1). Thus, on the basis of this genotyping data the size of the *Idd3* locus has been reduced to the 0.15-cM interval between, but not including, the markers *D3Nds36* and *D3Nds76* (Fig. 1).

Construction of a BAC Contig Across the *D3Nds36*-to-*D3Nds76* Interval

A BAC contig covering the minimal *Idd3* interval was constructed as follows. Insert ends of clones, identified by screening the mouse BAC library with the STSs described earlier, were isolated and used to develop new STSs. These new STSs were then used to rescreen the library, identifying additional clones that spanned the gaps between adjacent clusters of clones. A total of 24 clones were isolated that together span the interval between *D3Nds36* and *D3Nds76* (Fig. 2).

The size of each clone was determined by restriction enzyme digestion (Fig. 2). Each clone was digested independently with both *NotI*A and *SalI* to eliminate errors caused by the comigration of bands of similar size. STSs were assigned to individual *SalI* fragments by Southern hybridization and used to identify common

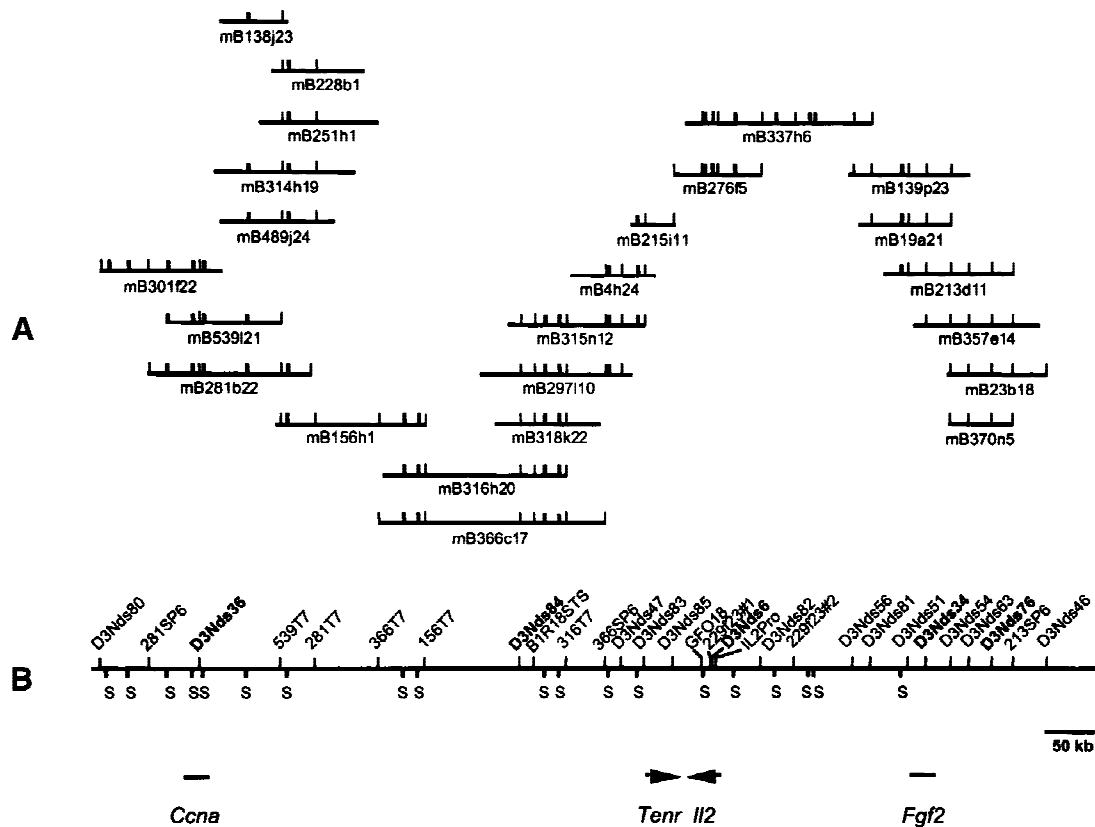


Figure 2 Physical map across the *Idd3* minimal interval. (A) *SalI* fingerprints of BAC clones spanning the interval. (B) *SalI* restriction map of the *Idd3* interval; markers were assigned to specific *SalI* bands by Southern hybridization. Markers shown in bold are polymorphic between NOD and B6. The gene content of the *Idd3* interval is shown below, where known the orientation of transcription is indicated by arrowheads (the transcription units are not drawn to scale).

bands in overlapping clones. Based on the degree of overlap between individual clones the physical distance between *D3Nds36* and *D3Nds76* is 780 kb (Fig. 2). The proximal boundary of *Idd3* lies in the 315-kb interval between *D3Nds36* and *D3Nds84*, whereas its distal boundary lies in the 65-kb segment between *D3Nds34* and *D3Nds76*. Thus, the size of the *Idd3* locus is between 400 and 780 kb (Fig. 2).

Refinement of the *Idd3* Interval by Haplotype Mapping

To refine the *Idd3* locus using congenic strains would be impractical given the large number of mice that would have to be bred to identify new recombinants within the interval. Therefore, to further narrow the interval we carried out haplotype analysis on six mouse strains known to carry either the *Idd3* susceptibility or protective allele. On the basis of either linkage analysis or congenic mapping the strains 129, SWR, and ABH have been determined to be susceptible at the *Idd3* locus, whereas the B6 and NON strains are diabetes resistant at the locus (Denny et al. 1997; Podolin et al. 2000).

These strains were genotyped for all the microsatellite markers and SNPs known to be variant between NOD and B6 that map to the 780-kb *Idd3* region (Table 1). In addition, nine more novel SNPs were identified in the six strains by sequencing STSs derived from BAC clone ends (Table 1). A comparison of the NOD and 129 mouse strains, both of which carry an *Idd3* susceptibility allele, shows that they have identical genotypes at all of the markers typed with the exception of *D3Nds36* (Table 1). This data confirms the congenic mapping data and identifies an *Idd3*-susceptible haplotype. The sequence identity between the two strains at multiple variable sites indicated the common ancestry of this segment of DNA between the NOD and 129 strains. Analysis of the two strains that carry an *Idd3*-protective allele, namely B6 and NON, reveals that they also have identical genotypes, but their shared ancestral haplotype is distinct from that found in susceptible strains at all markers (Table 1). This identifies an *Idd3*-protective haplotype and narrows the *Idd3* interval to the 365-kb region between *D3Nds84* and *D3Nds76*. Analysis of the ABH strain, which has an *Idd3*-susceptible allele, shows that it carries the *Idd3*-susceptible haplotype between the markers *229.1* and *D3Nds76* (Table 1). These data place the proximal boundary of *Idd3* in the 4-kb interval between the microsatellite marker *D3Nds6* and the SNP *229.1*. The SWR mouse strain has a recombinant haplotype; it has the susceptible haplotype at the markers *229.1* through *81.2* and the protective haplotype at the markers *81.3* through *51.2* (Table 1). Because SWR has an *Idd3*-susceptible allele this gives the most likely location of *Idd3* as the 145-kb region between, but not including,

the microsatellite marker *D3Nds6* and the SNP *81.3* (Table 1). As the G allele of the SNP *-694* is carried on both the protective and susceptible haplotypes it can be excluded as a candidate for the *Idd3* etiological mutation. Of the four genes known to map to the 780-kb interval, namely *Ccna*, *Tenr*, *Il2*, and *Fgf2*, only *Il2* maps to the 145-kb candidate region.

Analysis of *Il2* Gene Expression in Mouse Strains Carrying *Idd3* Susceptibility or Protective Alleles

The promoter regions of the *Il2* genes from NOD, B6, and 129 were sequenced to identify any polymorphisms that might lead to differences in transcription between mice carrying either *Idd3* susceptibility or resistance alleles. The B6 promoter sequence was determined by sequencing a cosmid clone, mC1h5, that contains the entire *Il2* structural gene. Based on this sequence data PCR primers were designed to amplify the entire *Il2* promoter region from nucleotide *-740* to nucleotide *+100*. This region has been shown previously to encompass all the regulatory elements required for transcription of *Il2* (Serfling et al. 1995). Using this PCR-based approach, the sequences of the NOD and 129 *Il2* promoters were determined and aligned to that of B6. Two polymorphisms were detected, one at position *-694* the other at position *-674*. In both cases the polymorphisms were A-for-G substitutions, with NOD and 129 having the A allele and B6 the G allele. In neither case did the polymorphism occur in a known transcription factor binding site.

To extend the evidence against the presence of functional regulatory allelic variants, the relative levels of transcription of the NOD, B6, and 129 *Il2* genes were measured. Total RNA from mixtures of either NOD and NOD.B6 *Idd3R450*, or NOD.B6 *Idd3R450* and NOD.129 *Idd3* splenocytes stimulated with ionomycin and PMA was used as the template for RT-PCR amplification with the microsatellite marker *D3Mit21*. *D3Mit21* amplifies a variant CAG repeat in exon 1 of *Il2*. The relative amount of RNA from each allele was quantified by analyzing the fluorescently labeled PCR products on an ABI 373 automated sequencer and calculating the ratio of the two products. As a control for preferential amplification genomic DNA was amplified with *D3Mit21* at the same time. No difference was observed in the steady state level of *Il2* RNA from either the NOD or the 129 allele compared with that from the B6 allele (ratio of NOD *Il2* RNA to B6 *Il2* RNA equals 0.89 ± 0.12 and ratio of 129 *Il2* RNA to B6 *Il2* RNA equals 1.06 ± 0.07 , Fig. 3). Taken together with the sequencing data this suggests that the reduction in diabetes conferred by *Idd3*-protective alleles is not due to differences in the level of transcription of the *Il2* gene.

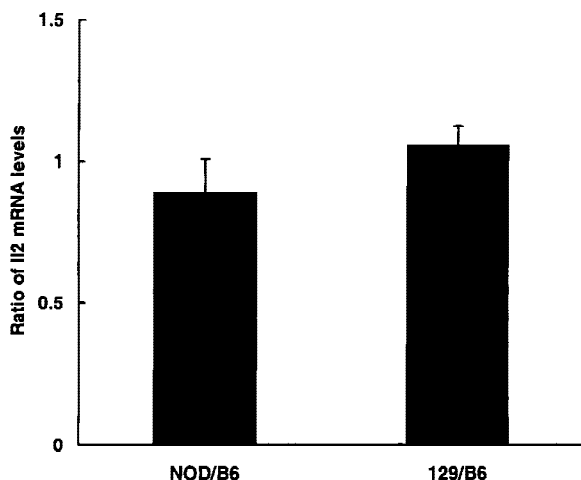


Figure 3 Transcriptional analysis of the *Il2* gene from mouse strains carrying either *Idd3* susceptibility or protective alleles. Relative *Il2* mRNA levels were determined by semiquantitative RT-PCR.

bility or protective allele at a particular locus carry the same allele. Given the observed degree of allele sharing IBD among the susceptible and resistant strains employed in the present study this assumption is likely to be valid for the *Idd3* locus. Given the size of the interval and marker density it is likely that the observed allele sharing is IBD and not merely identical by state (IBS). If the observed sharing were to be IBS it would invalidate this approach. Another assumption is that within the 0.15-cM interval there is only one locus responsible for the *Idd3* effect.

The *Il2* gene, the only gene currently known to lie within the 145-kb minimal interval, is a very strong candidate for *Idd3*. Mice that are deficient in the action of IL2, either through the targeted disruption of the *Il2* structural gene itself or the genes encoding the α or β chains of its receptor, develop a variety of autoimmune diseases (Hunig and Schimpl 1998). Studies in IL2 and IL2 receptor knockout mice have shown that the cytokine plays a nonredundant role during Fas-mediated apoptosis of activated T cells, one of the central processes in immune homeostasis (Van Parijs et al. 1997; Refaeli et al. 1998). Variants within the coding or regulatory elements of the *Il2* gene that alter the expression or function of IL2 would be strong candidates for the *Idd3* etiological mutation.

As described in this study we found no evidence of regulatory variants that alter the expression of the *Il2* gene, however, sequence variation has been described previously in the coding sequence of IL2 allotypes from different mouse strains (Chesnut et al. 1993; Ghosh et al. 1993; Matesanz and Alcina 1996, 1998; Denny et al. 1997). One sequence variant, a proline/serine substitution at position 6 of the mature IL2 protein, has been shown to correlate with diabetes suscep-

tibility or resistance at *Idd3*. Mouse strains carrying an *Idd3* susceptibility allele, such as NOD or 129, have a proline, whereas strains carrying an *Idd3*-protective allele, such as B6, have a serine (Denny et al. 1997; Podolin et al. 2000). We have shown recently that different IL2 allotypes have different glycosylation profiles and that this also correlates with the presence or absence of proline at position 6 (Podolin et al. 2000). Although these glycosylation differences have no effect on the ability of IL2 to stimulate proliferation of IL2-dependent cell lines (Podolin et al. 2000) it is conceivable that they may affect the half-life of the molecule leading to a functional deficiency in circulating IL2. It has been shown recently that IL2 can bind to heparan sulfate in vivo and that this bound IL2 is functional in both promoting T-cell activation and stimulating activation-induced cell death (Wrenshall and Platt 1999). This binding to heparan sulfate may well be altered by differences in glycosylation, thereby influencing in vivo availability of IL2.

Using a combination of congenic mapping and haplotype analysis we have narrowed the most likely location of *Idd3* to a 145-kb segment of DNA that contains the variant *Il2*. Although our genetic mapping data does not conclusively prove that the *Il2* gene is *Idd3*, the current lack of evidence for a functional difference between IL2 allotypes from mouse strains with *Idd3* susceptibility or protective alleles does not exclude it. Ultimately, the only definitive way to establish whether *Il2* is *Idd3* will be to construct a "knock-in" mouse in which *Il2* from NOD is replaced with the variant gene from a diabetes-resistant mouse such as B6.

METHODS

Animals

NOD/MrkTacfBR (NOD) mice were purchased from Taconic Farms, Inc. (Germantown, NY). The following congenic strains were used in this study: NOD.B6² (N11F2-3), NOD.B6³ (N11F2-3), NOD. B6 *Idd3*R450 (N13F2-3), NOD. B6 *Idd3*R808 (N13F2-3), and NOD.129 *Idd3* (N6F2-4). The derivation of congenic strains NOD.B6², NOD.B6³, NOD. B6 *Idd3*R450, and NOD.129 *Idd3* has been described previously (Lord et al. 1995; Denny et al. 1997; Podolin et al. 2000). The NOD. B6 *Idd3*R808 congenic strain was developed by backcrossing NOD.B6² to NOD and intercrossing the resulting F₁ progeny. The F₂ progeny were genotyped with markers within the *Idd3* interval and appropriate recombinants backcrossed to NOD. Suitable F₁ progeny were intercrossed to produce homozygous animals. The congenic strain was maintained by brother-sister mating. All mice were housed under sterile, specific pathogen-free conditions.

Assessment of Diabetes

Mice were monitored for the development of diabetes as described previously (Wicker et al. 1994).

STS Mapping and Isolation of Novel Microsatellites

Mouse BAC (Research Genetics) and P1 Imperial Cancer Re-

search Foundation libraries were screened by PCR according to the suppliers' instructions. Clone insert ends were rescued by vectorette PCR and STSs developed as described previously (Denny et al. 1997). Novel microsatellite markers were isolated from P1 clones using a PCR-based vectorette approach as described previously (Merriman et al. 1997). Primer sequences for the microsatellite and STS markers described in this paper are available on the web at <http://diesel.cimr.cam.ac.uk/todd>.

PCR Analysis

STS, fluorescent, and nonfluorescent PCR reactions were performed and analysed as described previously (Denny et al. 1997).

Restriction Enzyme Mapping of BAC Clones

BAC DNA was prepared using a standard alkaline-lysis protocol. Aliquots of 1 µg of BAC DNA were digested for 1 hr at 37°C with 10 units of either *NotI*A or *SalI*. Following digestion, DNA fragments were separated by pulsed-field gel electrophoresis. Gels were run at 200 V for 15.2 hr at 14°C with pulse times ramped from 0.2 sec to 21.8 sec. Following electrophoresis and visualization by ethidium bromide staining, DNA fragments were transferred to nylon filters by capillary action and fixed by UV cross-linking. Filters were hybridized with [γ -³²P]-labeled oligonucleotides.

Ii2 Promoter Sequencing

The cosmid mC1h5 was sequenced using a random shotgun approach essentially as described in Bankier et al (1987). Sequence data was assembled using the program GAP4 (Bonfield et al. 1995). This sequence data has been submitted to GenBank and assigned the accession number AF19596. Two sets of PCR primers were designed to amplify the *Ii2* promoter. IL2.Pro1 (5'-ATGAAAGTGCAACTAGAGCAC-3') and IL2.Pro3 (5'-GAGACACAAAAGTAACTCATG-3') give a 444-bp product spanning nucleotides 3773–4217 of the mC1h5 sequence and IL2.Pro2 (5'-CTTTTCATCTATCTCCTCTTGC-3') and IL2.Pro4 (5'-GACAAGGAGCACAAAGTGTCAT-3') amplify a 500-bp product spanning nucleotides 4115–4615. Following amplification, PCR products were gel purified and then sequenced, with the amplification primers, using an ABI Prism dye terminator cycle sequencing kit (PE Biosystems, Warrington, UK) according to the manufacturer's instructions. Sequence data for the NOD and 129 *Ii2* promoter regions have been submitted to GenBank and assigned the accession numbers AF19594 and AF19595, respectively.

Generation of IL2-Containing Splenocyte Cultures

One million mouse spleen cells were stimulated with 10 ng/ml of PMA and 400 ng/ml of ionomycin (Calbiochem, San Diego, CA) for 4 hr as described previously (Chen et al. 1994).

Quantification of *Ii2* mRNA Levels

Total RNA was extracted from 4×10^7 splenocytes stimulated with ionomycin and PMA as described above using an RNeasy RNA extraction kit (Qiagen, Crawley, UK) following the manufacturer's instructions. First-strand cDNA was synthesized from 1 µg of total RNA using a M-MLV H⁻ RT cDNA synthesis kit (Life Technologies, Glasgow, UK) according to the manufacturers instructions. The cDNA was diluted 1/100 and 1 µl was used as template for amplification with *D3Mit21* (primer sequences for this microsatellite marker are available on the web at <http://www-genome.wi.mit.edu/cgi-bin/>

mouse/index). Following amplification the fluorescently labeled PCR products were analyzed on an ABI373 automated sequencer as described previously (Denny et al. 1997). The amount of *Ii2* mRNA was quantified using Genotyper software (PE Biosystems).

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