Letter

Lymphopenia in the BB Rat Model of Type 1 Diabetes is Due to a Mutation in a Novel Immune-Associated Nucleotide (*Ian*)-Related Gene

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The BB (BioBreeding) rat is one of the best models of spontaneous autoimmune diabetes and is used to study non-MHC loci contributing to Type 1 diabetes. Type 1 diabetes in the diabetes-prone BB (BBDP) rat is polygenic, dependent upon mutations at several loci. *Iddm1*, on chromosome 4, is responsible for a lymphopenia (*lyp*) phenotype and is essential to diabetes. In this study, we report the positional cloning of the *Iddm1/lyp* locus. We show that lymphopenia is due to a frameshift deletion in a novel member (*Ian5*) of the Immune-Associated Nucleotide (IAN)-related gene family, resulting in truncation of a significant portion of the protein. This mutation was absent in 37 other inbred rat strains that are nonlymphopenic and nondiabetic. The IAN gene family, lying within a tight cluster on rat chromosome 4, mouse chromosome 6, and human chromosome 7, is poorly characterized. Some members of the family have been shown to be expressed in mature T cells and switched on during thymic T-cell development, suggesting that *Ian5* may be a key factor in T-cell development. The lymphopenia mutation may thus be useful not only to elucidate Type 1 diabetes, but also in the function of the *Ian* gene family as a whole.

[Sequence data reported in this paper has been deposited in GenBank and assigned the following accession nos: AF517674, AF517675, AF517676, and AF517677. Supplemental material is available online at http://depts.washington.edu/rhwlab/ and http:www.genome.org.] The following individuals and institutions kindly provided reagents, samples, or unpublished information as indicated in the paper: K. Matsumoto and the Sir Frederick Banting Research Centre.

Type 1 (insulin dependent) diabetes mellitus in humans is a significant health problem with a prevalence ranging from 0.3% to 1% in different populations (Onkamo et al. 1999). Genetic studies in the human, mouse, and rat have shown that there are many genetic factors contributing to Type 1 diabetes besides the major histocompatibility complex (MHC) (Nerup et al. 1974; Todd et al. 1987; Graham et al. 2002). In the diabetes-prone BB rat (BBDP), we have identified three loci contributing to Type 1 diabetes outside the MHC complex on rat chromosome 20; *Iddm1* on rat chromosome 4 (Jacob et al. 1992), *Iddm3* on rat chromosome 2 (Klaff et al. 1999), and a fourth factor on rat chromosome 15 (Kwitek et al. 2002). The *Iddm1/lyp* locus, linked to peripheral T cell lymphopenia (<15% normal T-cell count) and Type 1 diabetes, was mapped to a 0.7-cM interval on rat chromosome 4 (Jacob et al. 1992), and the genetic mapping has been replicated many times (Hornum et al. 1995; Kloting and Kovacs 1998; Klaff et al. 1999). One notable feature about the BBDP diabetes model is that lymphopenia is essential for the development of the diabetic phenotype and is inherited as a simple Mendelian trait (Jacob et al. 1992; Bieg et al 1998). To study the *Iddm1/lyp* locus in the absence of the other *Iddm* loci, we generated a congenic strain (DR.*lyp*), in which lymphopenia (*lyp*) and *Iddm1* from a line of inbred diabetes-prone BB rats (BBDP) (Eastman et al. 1991; Markholst et al. 1993) was introgressed onto the genome of inbred diabetes-resistant BB rats (BBDR) by marker-assisted selection (Bieg et al. 1998). This congenic rat strain has confirmed that a single locus is responsible for both T-cell lymphopenia and spontaneous autoimmune diabetes. In the completed congenic DR.*lyp* line, and in recombinant animals developed from this strain, no animal developed diabetes without lymphopenia (*lyp*) (Bieg et al 1998). This suggests that either pleiotrophy is responsible for both traits or that the lymphopenia gene is responsible for the loss of critical T cells, resulting in autoimmunity. Specific pathogen-free wild-type (+/+) and heterozygous (*lyp*/+) DR.*lyp*

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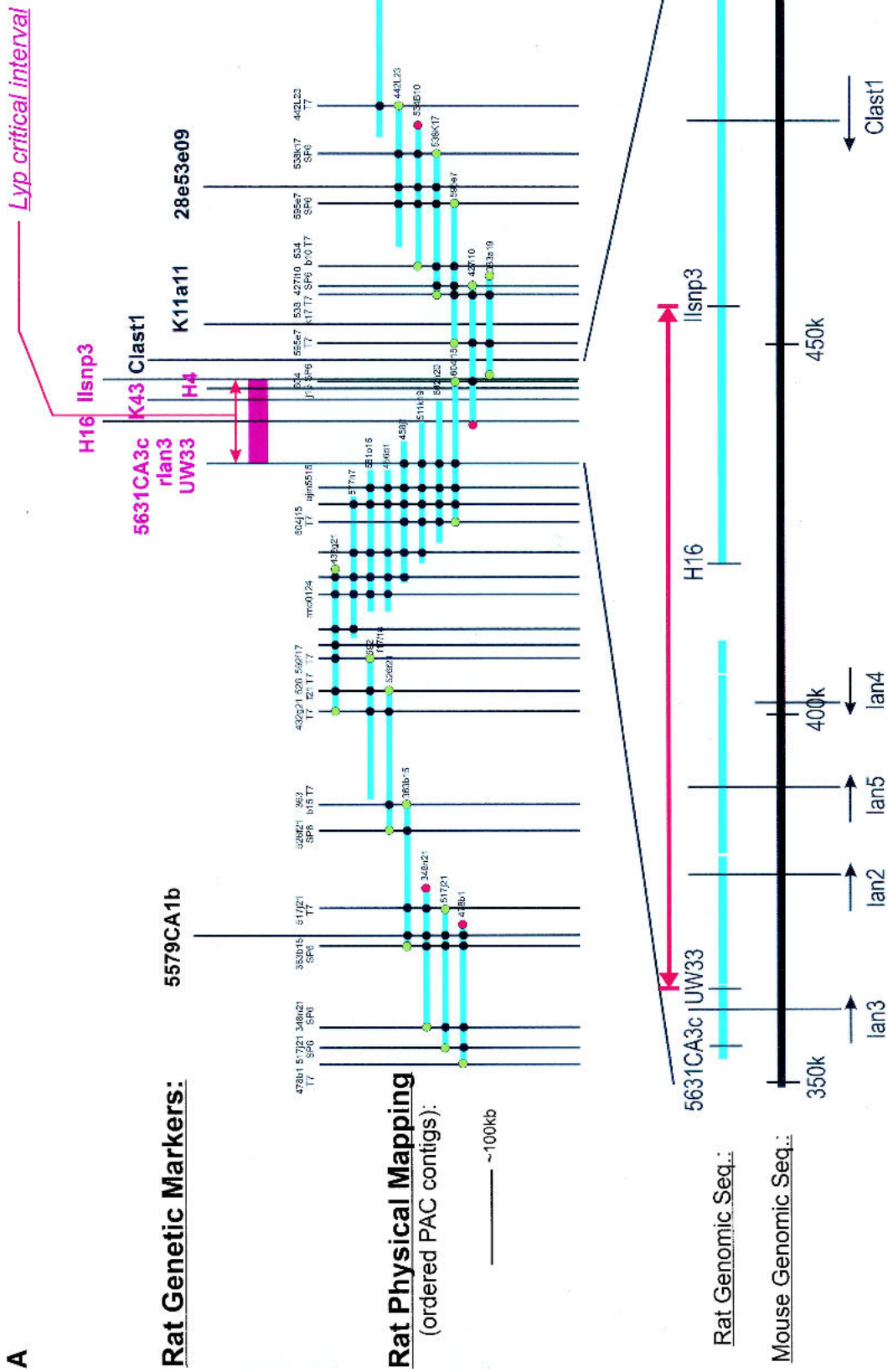

Figure 1 The lymphopenia gene region and the *lan5* transcript. (*A*) Physical map of the rat *lyp* gene region with genetic markers integrated (*top*). Overlapping PAC clones are shown along with the locations of genetic markers used to narrow the *lyp* interval, clone-end STS assays, and the limits of the *lyp* interval itself as a red arrow at both *top* and *bottom*. Distances between markers may not be strictly to scale because they are estimated on STS content. The *bottom* part shows an expanded view of the *lyp* interval, showing the locations of known genes and the extent of the assembled sequence contigs of rat genomic DNA, along with the framework of mouse genomic DNA sequence. A 13-kb-long rat genomic sequence contig includes the rat *lan5* gene. Position coordinates shown are those from mouse sequence supercontig Mm6_WIFeb01_100. (*B*) The cluster of *lan*-related genes. In human, this gene family is present on chromosome 7q36.1. In the mouse, it is located on proximal chromosome 6. We have also indicated the position of the human and mouse orthologs of the LR8/Clast1 (mouse accession no. AB031386) gene as location aids, although this gene is not in the IAN family. We have indicated various alternative names associated with each gene, and provisionally named previously unnamed members of the family as follows (these are indicated by underlines). For those genes without a common name, we have chosen to continue using the *lan* gene nomenclature. To avoid confusion, in this work we will refer to the genes in this family by using the name of the mouse ortholog and a prefix, h, m, or r, to specify which species is indicated (e.g., h*lan2* for the human ortholog of mouse *lan2*, otherwise known as *himap1*). Genes given the same *lan* designation in different species have been determined to be orthologs of each other. Genes with different designations do not show enough similarity to be deemed orthologs, with the exception of h*lan7*, which is orthologous to both m*lan7* and m*lan3*. Genes in species without a clear ortholog in the other have been given unique *lan* numbers (e.g., h*lan12*). Positions shown are within the respective contigs (accession nos. NT_007704.8|Hs7_7861 for human and supercontig Mm6_WIFeb01_100 for mouse). (*C*) Diagram of the rat, mouse, and human *lan5* gene transcripts, with exon structure shown to scale. Beneath each transcript diagram is a diagram of the extent of the major ORF (the *lan5*-coding region). Continued on the following page.

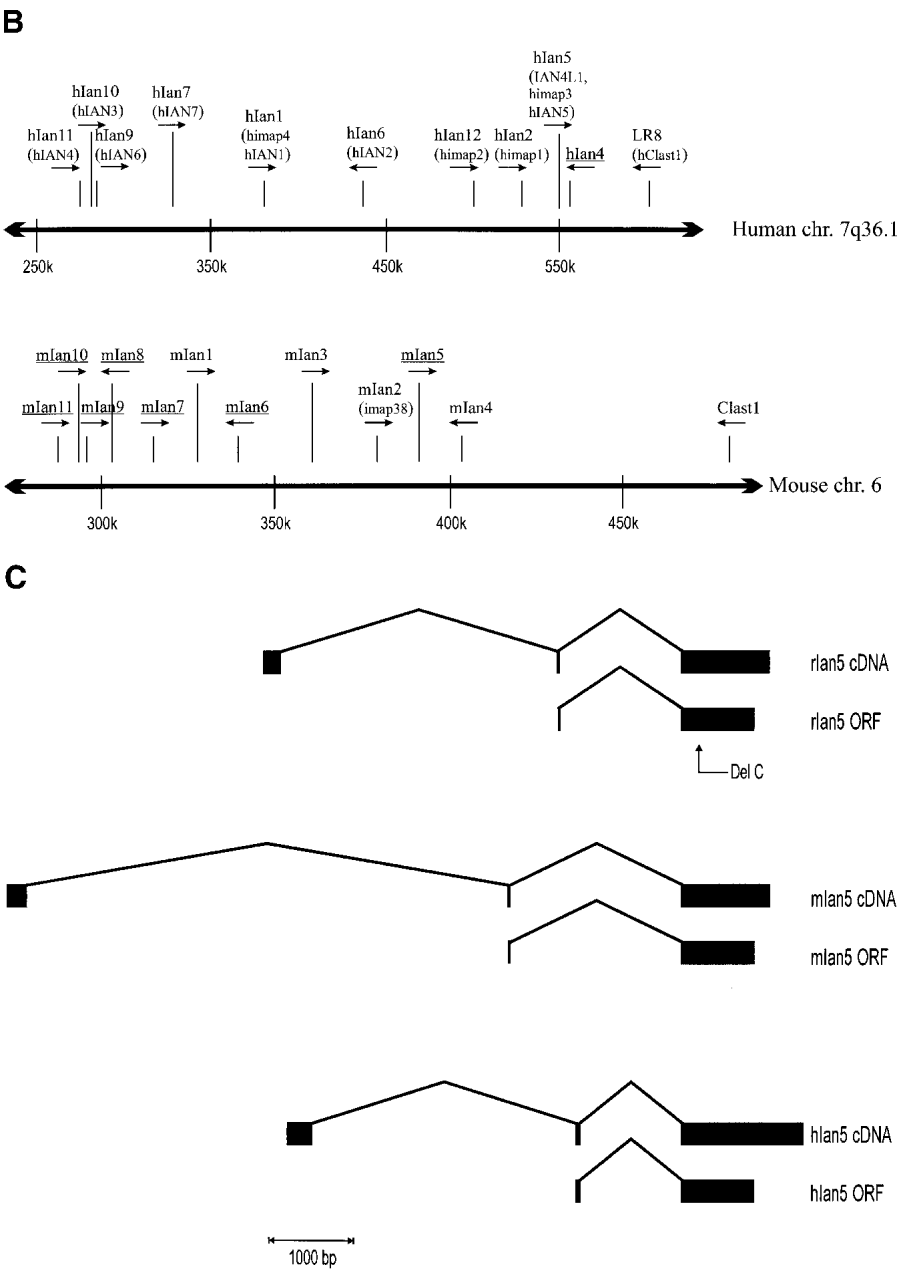

Figure 1 Continued

rats have normal lymphocyte numbers and do not develop diabetes, whereas DR.*lyp/lyp* rats have T-cell lymphopenia from birth and clinical onset of Type 1 diabetes between 50 and 108 days of age in 100% of the animals (Bieg et al. 1998; Klaff et al. 1999). The nature of the *Iddm1/lyp* gene is therefore critical to the understanding of age-dependent Type 1 diabetes development in the BB rat.

In the BBDP and DR.*lyp* strains, *Iddm1/lyp* is a single Mendelian trait; therefore, we set out to identify it by using a positional cloning approach. We generated genetic and physical fine-structure maps of the region to identify and evaluate positional candidate genes for *Iddm1/lyp*. Here, we report the identification of a single nucleotide deletion in a novel member, *Ian5*, of the immune-associated nucleotide gene family, resulting in a largely truncated protein.

RESULTS

Comparative Genomics of the *Iddm1* Genomic Interval

When we began the positional cloning of *Iddm1/lyp*, physical maps were not available for the rat. Therefore, we began by constructing a physical map of the syntenic region in the mouse, with the expectation that the mouse ortholog of *Iddm1/lyp* would lie within this interval. Comparative mapping determined that the *Iddm1/lyp* region on rat chromosome 4 (between *D4Mit6* and *D4Mit24*) is syntenic to the proximal end of mouse chromosome 6 (*Mmu6*). Gene order appears to be conserved between rat and mouse, over the region just proximal of the rat *Iddm1/lyp* region (including the T-cell receptor β-chain genes) to 10–15 cM distal of the locus (including the immunoglobulin κ chain complex, *Igk*) (data not shown). Because we expected the mouse *Iddm1/lyp* region to contain the ortholog of the rat *Iddm1/lyp* gene, we set out to isolate the genomic DNA of both the mouse and rat *Iddm1/lyp* regions, combining the information and reagents from both species to create a comprehensive map of the region.

We initially constructed a mouse YAC contig spanning the ~2-Mbp interval of the mouse *Iddm1/lyp* region, and isolated gene fragments from that interval. We then used the mouse gene fragments as probes to isolate the corresponding orthologous rat gene fragments by cross-species cDNA selection (Lovett et al. 1987). Next, we constructed a rat YAC contig spanning the rat *Iddm1/lyp* region by isolating those rat YAC clones that contained the rat gene fragments. STS content mapping and hybridization of gene fragments from one map to the other confirmed that the local gene order was the same in rat and mouse (these and other supplementary data are available on the authors' web site http://depts.washington.edu/rhwlab/ and at http://www.genome.org.).

Characterization of the Rat, Human, and Mouse *lyp* Regions

The initial mouse physical map was converted into a more useful higher-resolution form by isolating contigs of genomic

BAC clones. We sequenced overlapping mouse BAC clones spanning >800 kb of the mouse *Iddm1/lyp* region. STSs from these BAC clones were then used to refine the rat physical map by identifying corresponding rat PAC clones (rat contig shown in Fig. 1a), which were then sequenced.

While generating the physical map of the *Iddm1/lyp* regions in mouse and rat, we also generated recombinant animals to refine the position of *Iddm1/lyp* on the rat genetic map (Table 1). We continued to intercross BBDR (+/+) and DR.*lyp* (*lyp/lyp*) rats, as well as to backcross and then intercross DR.*lyp* (*lyp/lyp*) and F344 rats. These crosses provided >300 additional animals in addition to the ~870 animals already analyzed (Jacob et al. 1992), totaling over 2400 meioses. Resulting recombinant animals identified the *Iddm1/lyp* interval, flanked by an SSLP, UW33, on the proximal end and a SNP, *IIsnp3*, on the distal end (Fig. 1a). This region corresponds to ~100 kb on the mouse genome.

With the recent assembly of a draft sequence of the mouse genome by the International Mouse Genome Sequencing Consortium (http://www.ensembl.org/Mus_musculus; Batzogloo et al. 2002), and other draft mouse contigs (e.g., MGSCv3 as available at http://ncbi.nlm.gov/genome/seq/MmBlast.html) we integrated our sequence to produce a contig including the entire mouse region orthologous to the rat *lyp* interval (Fig. 1b). We then aligned the mouse genomic sequence to the human syntenic region on chromosome 7q36.1 and evaluated the conserved genes annotated in both species.

A notable feature of this region is the presence of a family of at least 10 putative GTP-binding protein genes found only in this region of the human and mouse genomes, the Immune Associated Nucleotide (IAN) gene family (Krucken et al. 1999; Poirier et al. 1999; Daheron et al. 2001; Cambot et al. 2002; Stamm et al. 2002). Interestingly, all *Ian* gene family members are located in a 300-kb interval within 7q36.1 and a more compact 120-kb region in the mouse. This may be a consequence of genomic rearrangement in the human *Ian* gene region relative to the mouse *Ian* gene region, because of the two species' evolutionary divergence, as the number of *Ian* genes differs in each (10 in human, 11 in mouse), and there are breaks in the gene order of the orthologs between the species (for example, h*Ian12* has no ortholog in the mouse, and m*Ian3* is one of two orthologs of h*Ian7*). We examined the region in the mouse genome corresponding to the critical *Iddm1/lyp* interval in rat and found that three IAN family members lay within this critical region (Fig. 1a). Whereas *Ian2* was expressed in the spleen (Krucken et al. 1997), *Ian4* was only expressed at low levels but was not detected in any other lymphoid tissue (Daheron et al. 2001). The third gene, designated *Ian5*, has not been reported previously in mouse or rat. Interestingly, rat *Ian3* is differentially expressed in thymus and spleen when comparing tissue from DR.*lyp*+/+ and *lyp/lyp* rats (data not shown), but was excluded as a candidate because it lay outside of the critical region.

Identification of r*Ian5* that Contains a 1-bp Deletion Unique to the DR.*lyp* Rat

The intron/exon structure of the r*Ian5* gene is shown in Figure 1c in comparison with its mouse and human orthologs, m*Ian5* and *IAN4L1* (*hIan5*). The overall genomic structure is similar to that reported previously in this family of genes (Stamm et al. 2002). As with h*Ian5*, r*Ian5* has at least three exons. The first and second exons are short, 220 and 49 bp, respectively, whereas the last exon is 1047 bp. There is a 3895-bp intron between exons 1 and 2, and a 1457-bp intron between exons 2 and 3. Exon 2 contains the putative start site for the major ORF spanning exons 2 and 3, as reported previously for m*Ian4* (Daheron et al. 2001). Exons 1 and 2 contain an additional 61 amino acid ORF starting at position 78; this overlaps the major ORF and has no significant amino acid sequence similarity with the small 5′ ORF in *mIan4*.

We established that r*Ian5* is expressed in rat spleen, thymus, and lymph nodes, making it a strong positional candidate for *Iddm1/lyp* in the BB rat. To identify potential functional variants in this gene in the DR.*lyp* rats, we used primers spanning the putative coding sequence to amplify and sequence the gene from both BBDR wild-type and DR.*lyp* (*lyp/lyp*) thymic cDNA. The cDNA sequence was confirmed by sequencing of BBDP/WorAp and BBDR as well as F344 rat genomic DNA (Fig. 2). The sequence analysis showed that both the DR.*lyp* and BBDP/WorAp strains lack one C nucleotide at basepair position 478 of r*Ian5*, causing a frameshift mutation in the presumed ORF (exon 3) and leading to a significantly truncated predicted protein product (Fig. 3). The frameshift deletion in the *lyp/lyp* r*Ian5* changes the predicted downstream amino acids to include 19 amino acids (boxed) before the premature STOP codon (Fig. 3). We confirmed that this nucleotide deletion was present in our lymphopenic congenic F344.*lyp* inbred rat line (data not shown) as well as in outbred BBDP (diabetes prone) rats from Ottawa (Table 2). As expected, the nonlymphopenic, diabetes-resistant outbred BBDR rat from Ottawa did not contain this deletion.

Table 1. Recombinant Genotypes Specify the Boundaries of the *lyp* Critical Interval in the Rat

Cross	Rat	5631CA3c	UW33		H16	K43	H4	*lyp*	IIsnp3		Clast1		K11a11		28e5e09
BB-DP × BB-DR	11CF2 19:2	3	3		3	3	3	+	nt		3		3	X	1
	2CF3 1:12	1	1		nt	nt	1	Lp	nt	X	nt		nt		3
BB-DP × F344	1FBF2 18:6	1	1		1	1	1	Lp	111 × 33		3		3		3
	2FBF2 11:5	1	1		1	1	1	Lp	11111	X	3		3		3
	4FBF2 14:13	3	3		3	3	3	+	33333		3	X	1		1
	4FBF2 31:11	3	3	X	1	1	1	Lp	11111		1		nt		1

The type of cross and the identification of the recombinant animals are listed to the *left* and the genotypes (or phenotypes in the case of *lyp*) at selected loci are shown. The key recombinant animals defining the *lyp* critical interval H16-1Isnp3 are 4FBF2 31:11 on the *left* (proximal) side and 1FBF2 18:6 on the *right* (distal) side. The X marks indicate the inferred locations of recombination breakpoints, nt indicates genotypes not tested. Lp is lymphopenic (the cut off value is 15%, representing the mean + 14SD) and + indicates a normal (non-*lyp*) phenotype. SNP marker IIsnp3 is not polymorphic in the BBDP × BBDR cross.

To determine whether the frame shift deletion was a common polymorphism among rat strains or mutation unique to strains with lymphopenia, we resequenced ~500 bp of r*Ian5*, encompassing the deletion, in 38 rat strains (Table 2). The different strains have been characterized with genetic markers spanning the genome and were selected to represent inbred lines or strains of rats with maximum genetic diversity (Steen et al. 1999); only the BBDP/Ottawa and BBDP/WorAp strains have lymphopenia and Type 1 diabetes. The frameshift mutation was found only in the strains with lymphopenia (BBDP/Ottawa and BBDP). Three other sequence variants were found among the 38 strains and can be summarized as three distinct haplotypes (Table 2). The most common haplotype was found in 26 of the 38 strains; the frameshift mutation occurs on this haplotype. Whereas the normal r*Ian5* sequence predicts a protein of 35 kD, the deletion mutant would represent a dramatically truncated protein of 11 kD.

rIan5^{del} Expression Reduced in Hematopoetic Cells

Northern blot analysis of poly(A$^+$) RNA prepared from DR.+/+ and *lyp/lyp* rat tissues showed that the r*Ian5* transcript of 1.4 kb was expressed in the thymus and spleen but not in the kidney (Fig. 4a). The transcript levels were markedly reduced in *lyp/lyp* as compared with +/+ tissue. *Ian5* mRNA levels in thymus of *lyp/lyp* animals were reduced to 45% of wild-type levels. The level was even lower in the spleen of *lyp/lyp* animals (only 6% of wild-type levels), although this may reflect the absence of T cells resulting from the lymphopenia phenotype. Expression levels in the kidney were extremely low (3% of wild-type thymus), and differences between phenotypes could not be reliably observed. To prove that the decreased expression levels were a direct result of the frameshift mutation and not a secondary consequence of the absence of T cells due to lymphopenia, we examined expression levels in *lyp/+* heterozygotes that show no lymphopenia. Heterozygotes showed intermediate levels of expression (Fig. 4b). These data support the notion that the frameshift mutation in the r*Ian5* gene causes a marked reduction in the mRNA in hematopoietic tissues established previously to be affected by T-cell lymphopenia and results in the lymphopenia and diabetes in the diabetic-prone BB rat. The reduced levels of r*Ian5* transcripts found in *lyp/+* heterozygotes cannot be explained on the basis of T cell numbers, as both wild-type and heteozygotes have normal numbers of thymocytes and peripheral T cells, but may rather be due to the possibility that the mutated *rIan5* transcripts are unstable.

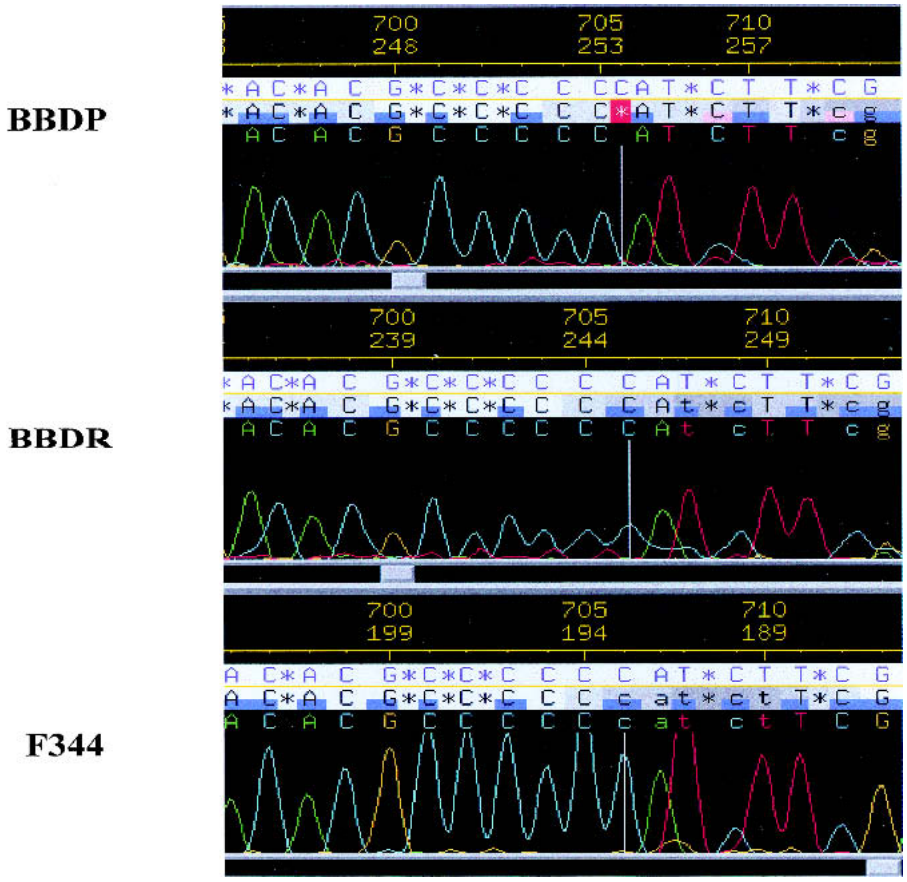

Figure 2 Sequence of the BB rat Immune Associated Nucleotide (*Ian5*) gene. A representative sequencing trace of DNA from BBDP/WorAp compared with wild-type BBDR/WorAp and F344 rats. The frameshift mutation at nucleotide position 478 in the DP rat DNA is indicated. DNA sequences were determined on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) and analyzed by the use of `Phred`, `Phrap`, `Consed`, and `PolyPhred` for sequence assembly and identification of sequence variants.

DISCUSSION

The BB rat has many important features as a model for the study of Type 1 diabetes (Crisá et al. 1992; Pettersson et al. 1996), including the presence of a simple mendalian trait, lymphopenia, which is absolutely essential for diabetes. In this work, we have shown that lymphopenia is due to a mutation in the novel rat *Ian5* gene.

Ian5 belongs to a new and largely uncharacterized family of GTP-binding proteins that share sequence homology with the gene encoding the pathogen-induced plant protein *aig1* (Reuber and Ausubel 1996). In *Arabidopsis*, this gene is induced after infection with *Pseudomonas syringae*. Another member of the *Ian* family, *IAP38*, was induced in mice by blood-stage infections of *Plasmodium chaubadi* malaria (Krucken et al. 1997) and reported to be expressed in splenic macrophages, B cells, and T cells, providing some functional evidence that this family of genes plays a role in immune response.

The wild-type (DR) sequence of r*Ian5* predicts a protein with 308 amino acids, which is 80% identical to m*Ian5* and 52% identical to h*Ian5* (Daheron et al. 2001; Stamm et al. 2002). In the mouse, the coding regions of m*Ian5* and m*Ian4* are 80% identical, although the noncoding portions of the genes are not

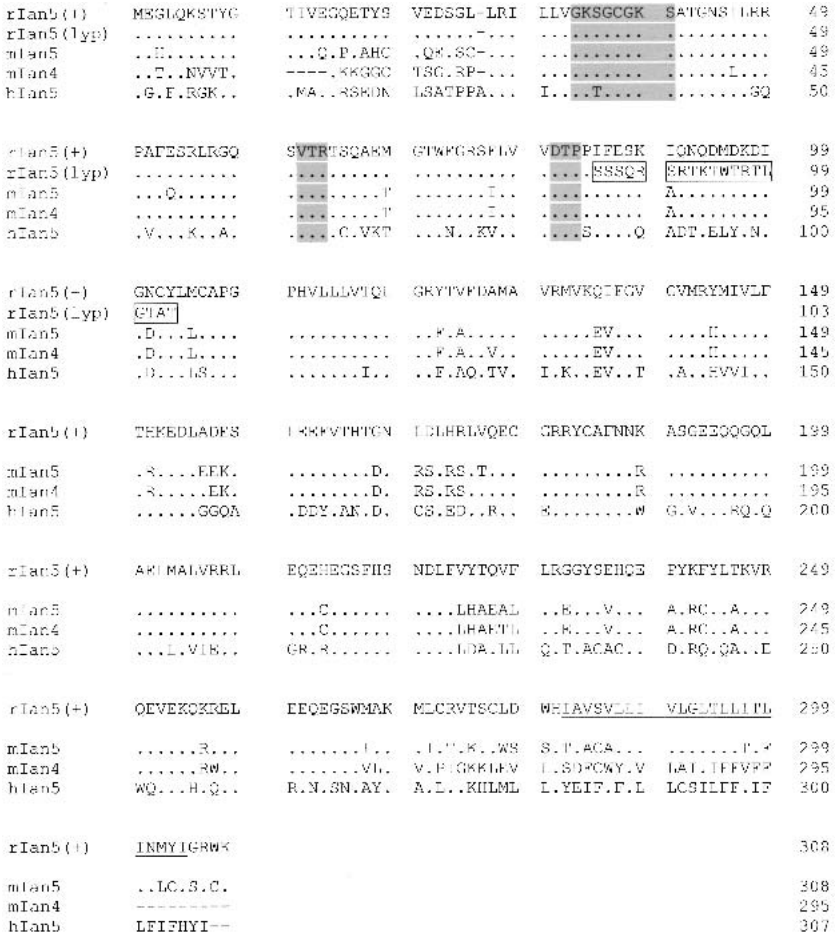

Figure 3 Sequence comparisons between BBDR wild-type (+/+), *lyp/lyp* rat, mouse, and human Immune Associated Nucleotide-5 (*Ian5*) and mouse *Ian4* predicted amino acid sequences. The deletion of a nucleotide in the codon for amino acid 85 of the r*Ian5* (*lyp*) changes the predicted downstream amino acids to include 19 amino acids (italicized) before the premature STOP codon at amino acid position 104. Putative ATP/GTP-binding sites are boxed/shadowed and a hydrophobic putative transmembrane region underlined.

substantially similar. In fact, amino acids 23–262 in m*Ian4* and amino acids 27–266 in m*Ian5* show only 7 amino acid differences (Fig. 3). The three species share an ATP/GTP-binding site at position 33–40 (Saraste et al. 1990). Other conserved regions for GDP/GTP exchange, GTP-induced conformational change, and GTP hydrolysis (Bourne et al. 1991), as suggested previously (Poirier et al. 1999; Cambot et al. 2002; Stamm et al. 2002), were identified at amino acids 61–63 and 81–83. A transmembrane domain at 282–304 was strongly predicted by TMHMM (Krogh et al. 2001).

The understanding of the genomic organization of this family of genes, as well as the function of the proteins, is as yet fragmentary as is the information about which cells express these proteins, and their subcellular localization. Apart from analyses in a variety of cell lines (Daheron et al. 2001), several of the *Ian* proteins are thought to be expressed in T or B cells, as well as in macrophages and dendritic cells. In contrast to our data on the rat *Ian5* gene expression, mouse *Ian4* was only expressed at low levels in the spleen and no expression was found in the thymus, liver, and kidney (Daheron et al. 2001). Mouse *Ian5* has not been studied previously. Further studies are therefore required to identify the specific cell types in the BB rat that express the *Ian5* protein. The faint expression in the kidney may reflect *Ian5* expression in T cells.

The marked reduction in r*Ian5* expression in DR.*lyp/lyp* lymphoid organs may have several explanations. Lymphopenia in the BB rat is associated with a marked reduction in blood lymphocytes (Jackson et al. 1981; Bellgrau et al. 1982; Poussier et al. 1982; Elder and Maclaren 1983; Guttmann et al. 1983; Plamondon et al. 1990; Eastman et al. 1991) affecting both $CD4^+$ and $CD8^+$ T cells (Crisá et al. 1992), but in particular, T cells with the RT6 (ART2) T-cell marker (Greiner et al. 1997). Clearly, the reduction in T cells affects primarily peripheral blood, spleen, and lymph nodes, but not thymus (Gold and Bellgrau 1991; Bieg et al. 1997; Hernandez-Hoyos et al. 1999). The BBDP rat also exhibits abnormalities in the thymic T-cell subset (Groen et al. 1996), epithelial cell distribution (Doukas et al. 1994), and in vitro T-cell development (Whalen et al. 1999). The possibility that *Ian5* is expressed chiefly in T cells is supported by the drastic reduction in expression in DR.*lyp/lyp* lymphoid organs. In this case, the observation that heterozygous DR.*lyp/+* rats had levels of *Ian5* that were intermediate between *lyp/lyp* and +/+, but had normal T-cell levels, is best explained by the possibility that the mutated r*Ian5* transcript is unstable.

It will now be possible to determine whether the truncated *Ian5* is causing lymphopenia because of the loss of a transmembrane domain or because of a greater functional disruption to the protein. Future studies can also test the hypothesis that *Ian5* may be a key factor in T-cell development. It will also be possible to begin the analysis of pathways downstream of *Ian5* to uncover important checkpoints in the emergence of age-dependent autoimmune diabetes. All of this will greatly enhance the utility of the BB rat in dissecting mechanisms that control autoimmunity.

Finally, it will now be feasible to test whether this gene plays a role in human Type 1 diabetes by identifying SNPs in the gene and conducting association studies. Independent of the role this gene plays in human diabetes, its identification will increase our understanding of both the disease process and the role of non-MHC loci in diabetes.

METHODS

Rats

BBDR (Bieg et al. 1998) and F344 rats (Klaff et al. 1999) congenic for lymphopenia were maintained at the University of Washington. All animals were kept under specific pathogen free (SPF) conditions with standard light-dark cycles. The rats were fed a regular diet. Sentinel animals were negative for

Table 2. Sequence Analysis of *Ian5* in Different Inbred Strains of Rats

	Position in r*Ian5*		
Strain	378	472	478
BBDP/WorAp	C	G	*
BBDR.+/+	C	G	C
BB DP/Ottawa	C	G	*
BB DR/Ottawa	C	G	C
ACI	C	G	C
BN/Ssn	C	G	C
DRY	C	G	C
F344	C	G	C
FHH	C	G	C
GK	C	G	C
LEA	C	G	C
LEC	C	G	C
LEW	C	G	C
LH	C	G	C
LN	C	G	C
MNRA	C	G	C
MR	C	G	C
NEDH	C	G	C
ODU	C	G	C
OKA	C	G	C
OLETF	C	G	C
P	C	G	C
SD	C	G	C
SHRSP	C	G	C
SRJR	C	G	C
SSJR	C	G	C
WAG	C	G	C
WF	C	G	C
WN	C	G	C
LE	C	A	C
M520	C	A	C
WTC	C	A	C
WIST	C	A	C
WKAH	C	A	C
BUFF	G	G	C
DA	G	G	C
MNR	G	G	C
NP	G	G	C
OM	G	G	C

Inbred strain designations and descriptions can be found on RGD (http://rgd.mcw.edu). Genotypes are listed at the specific position in rIan5. Yellow highlighting represents the most common allele. Blue highlighting represents the less common SNP. The deletion found in the diabetic prone BB rat is designated by *.

viral antibodies and parasites during the period of the experiments. Siblings heterozygous for polymorphic markers flanking the lymphopenia interval were used as breeding pairs to generate homozygous animals. The rats were screened for diabetes and lymphopenia as described in detail (Bieg et al. 1998).

DNA was obtained from 32 different rat strains as described previously (Kwitek et al. 2001). In addition, we analyzed DNA from LEA, LEC, OLETF, and WKAH rats kindly donated by Dr. Kozo Matsumoto and from outbred BBDR and BBDP rats from Health Products & Food Branch, Sir Frederick Banting Research Centre, .

Physical Mapping and STS Screening

Mouse YAC Contig

Mouse YAC contigs were generated by first screening with known STSs and then filling in gaps by sequencing YAC ends and using resulting nonrepetitive sequence as additional STSs. Unless pre-existing or otherwise noted, PCR primers were selected using the `Primer 0.5` program (Lincoln et al. 1991) to choose primers with predicted melting temperatures within 1°C of 60°C and to avoid regions with repeat- or self-similarity. PCR amplification was performed according to the conditions specified for each protocol, or as described previously, or, if not specified, according to standard conditions as recommended by Perkin-Elmer.

YACs were isolated from the MIT mouse YAC library (Kusumi et al. 1993) by use of standard PCR screening methods and the YAC DNA prepared as described (Segre et al. 1995). YAC ends were isolated by use of inverse PCR as described previously (Haldi et al. 1994) and sequenced directly by use of standard fluorescent sequencing methods.

Higher Resolution Physical Mapping

Mouse bacteriophage P1 clones were isolated from two libraries, the P1 mouse RIII (2–3× coverage) and the P1 mouse ES (3× coverage) libraries (Pierce et al. 1992; Sternberg et al. 1994) (Genome Systems). Mouse BAC clones were isolated from a 129/SV mouse BAC library CITB CJ7B prepared by Bruce Birren (7× coverage) (Shizuya et al. 1992; Kim et al. 1996). Rat PAC clones were isolated from the RPCI-31 library (Woon et al. 1998). Each library was screened by a PCR-based or hybrid PCR- and hybridization-based protocol, as recommended by the library maker. P1, BAC, and PAC DNA was prepared according to standard protocols and as recommended by Genome Systems. P1, BAC, and PAC end sequences were obtained by use of a protocol similar to that for cloning YAC ends. STS content maps were assembled by use of standard PCR techniques to determine the STS content of panels of miniprep DNA from the isolated P1s, BACs, and PACs. Small rat genomic contigs were assembled by sequencing subclones, by directly sequencing PCR products, and by assembling contigs from the publicly-available rat genomic raw trace (WGS) database maintained by NCBI (http://ncbi.nlm.nih.gov/genome/seq/RnBlast.html).

Cross-Species cDNA Selection

Cross-Species cDNA Selection was performed by use of a protocol modified from that described previously by Lovett (1987, 1991) and the primers cDNA-1 (5′-CTGAGCGGAATTCGTGAGACC-3′)/cDNA-2 (5′-P-GGTCTCACGAATTCCGCTCAGTT-3′). All mouse template cDNAs were separately PCR amplified 10–15 cycles (94°C/64°C/72°C) with the bio-cDNA-1 primer (5′-biotin-CTGAGCGGAATTCGTGAGACC-3′; 64.4°C predicted melting temperature) and purified. Double-stranded rat cDNA from testis and spleen with an average fragment size of ~500 bp was modified with linkers com-

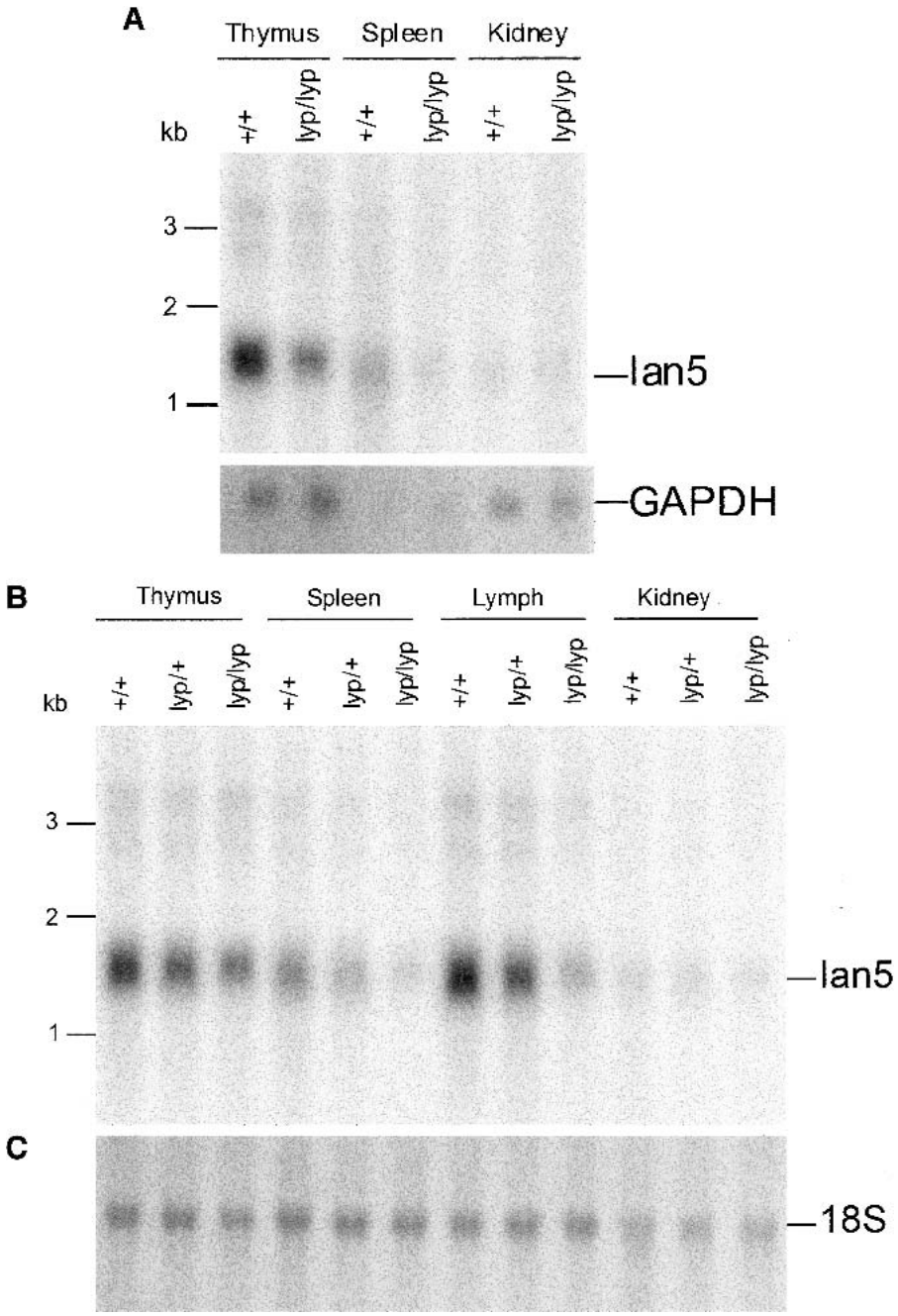

Figure 4 Expression of rat *Ian5* in tissues from *lyp/lyp*, lyp/+, and +/+ DR BB rats. (*a*) Northern blot containing 3 µg of poly(A$^+$) RNA from thymus, spleen, or kidney of each of +/+ or *lyp/lyp* rats probed with a 695-bp region of *Ian5* showing a 1.4-kb transcript (*Ian5*). The blot was stripped and reprobed with a 1420-bp GAPDH probe (GAPDH). The images were quantitated using a PhosphorImager and software and normalized to GAPDH expression in each lane. Size markers are indicated. (*B*) Northern blot containing 3 µg of poly(A$^+$) RNA from thymus, spleen, lymph node, and kidney from each of +/+, +/*lyp*, and *lyp/lyp* rats probed as in *A*. Size markers are indicated. (*C*) Methylene blue stain of the blot in *B* before probing, showing even loading of 18S ribosomal RNA in each lane.

posed of the two oligos cDNA-1b (5′-GTCACGCAAGCTTCT CACAGG-3′) and cDNA-2b (5′-P-CCTGTGAGAAGCTTGCGT GACTT-3′) and amplified using the cDNA-1b primer. A total of 1 µg-amplified cDNA, 2 µg mouse C_0t-1 DNA (BRL), and 2 µg glycogen (BMB) were prehybridized to a C_0t value four times greater than in the standard protocol. The prehybridized rat cDNA mixture was then mixed with the mouse template cDNA and hybridized essentially as in the standard protocol. After hybridization was stopped, the biotinylated material was washed 3 × 15 min in 0.1 × SSC/0.1% SDS at one of the three wash temperatures 65, 55, or 50°C (depending on the stringency desired). Finally, the selected cDNAs were eluted and eventually dU-cloned into the pAMP10 vector (BRL) by amplifying 30 cycles with 60°C annealing using the cDNA-U-2 primer (5′-CUA CUACUACUA GTCACGCAAGCTT CTCACAG-3′).

Genotyping

DNA was extracted from rat tail biopsies obtained at 25–30 days of age. Genotyping for simple sequence repeat markers was carried out as described previously (Jacob et al. 1992). Rat tail DNA was PCR amplified using IRD-700 tailed primers (LI-COR Biosciences). The PCR products were analyzed using a NEN Global IR^2 DNA Analyzer System (Model 4200S-2) using 6.5% gel matrix (LI-COR Biosciences).

DNA Sequence Analysis

Initial Sequence Analysis of BB DR.lyp

Primer pairs were designed for amplification of the *rIan5*-coding exons 2 and 3 (forward primer, 5′-GCTTGAGGAGGTCATCAGTTC-3′ and reverse primer, 5′-CTCACGTC CCAGCCTCTAAC-3′). PCR reactions were 2 min at 95°C; 10×; 30 s at 95°C, 30 s at 60°C, 30 s at 72°C; 30× 30 s at 95°C, 30 s at 60°C, 30 s plus 10 s/cycle at 72°C; 7 min at 72°C. The PCR products were purified with Ultrafree-MC (Millipore). We subjected purified PCR products (30–60 ng) to cycle sequence reactions using IRDye 800 terminators (LI-COR) and Thermo Sequenase (USB). The reaction products were purified with a MultiScreen Filtration System (Millipore) using Sephadex TM G-50 Fine (Amersham Pharmacia Biotech) and analyzed using NEN Global IR^2 DNA Analyzer System sequencer (LI-COR Inc.)

Sequencing of Additional Inbred Rat Strains

Primers were selected for PCR amplification of 500 bp (forward primer 5′-CCATGGCTTTGAGGAACTATCC-3′ and reverse primer 5′-TGTGGGTGAAGAGGACAATCAT-3′) and 385 bp (forward primer 5′-AAAGTGCCACAGGGAACAGC-3′ and reverse primer 5′-GTGTGGGTCACAAACTCTTCCA-3′) frag-

ments, encompassing the *rIan5* deletion mutation. Amplified products were subject to standard fluorescent sequencing using an ABI3700 automated sequencer. Analysis was performed using `Phred`, `Phrap`, `Consed`, and `Polyphred` to compare the sequences between BBDP and 38 other inbred rat strains.

In Silico Sequence Analysis

For the human, we used NCBI's genomic `TBLASTN` with the predicted protein product of h*Ian5* blasted against the GenBank human genome as of 12/24/01, setting the expectation parameter to 10 (http://ncbi.nlm.nih.gov/genome/seq/page.cgi?F=HsBlast.html&&ORG=Hs). The E values of the resulting matches were bimodal, with the matches plotted in Figure 1b ranging from ê-167 to 4ê-28 and the remaining spurious matches having E values >1.

For the mouse, we again used `TBLASTN` with the predicted protein product of m*Ian4* blasted against the GenBank mouse genome supercontig database (mgscv3) posted on 4/19/02, setting the expectation parameter to 10 (http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html). Again, the resulting E values were bimodal, with the Figure 1b matches ranging from ê-160 to 3ê-8 and the remaining spurious match having an E value of 1.5. We also used other NCBI `Blast` programs such as `BlastN` and `Blast2` according to recommended settings, to identify homologous EST sequences, already-identified genes, and the alignments of one sequence within another (Altschul et al. 1990, 1997); http://www.ncbi.nlm.nih.gov/BLAST/.

RNA Isolation and Quantification of *Ian5* mRNA Levels

Organs were dissected from 49-day-old congenic DR.*lyp* rats of each of three genotypes (wild-type or +/+, lyp/+, and *lyp/lyp*) from the same litter and homogenized immediately. Poly(A^+) RNA was isolated using QIAGEN RNeasy and Oligotex kits (QIAGEN). A total of 3 µg of poly(A^+) RNA per well was electrophoresed through a 0.9% agarose gel containing MOPS buffer [40 mM 3-N-morpholino-propane sulfonic acid (MOPS), 10 mM sodium acetate, 1 mM EDTA), and 2% formaldehyde. The gel was washed twice, 30 min in DEPC water, 35 min in 50 mM NaOH, 1.5 M NaCl, 30 min in 1 M Tris (pH 8.0), 1.5 M NaCl, and 5 min in 10× SSC (1× SSC is 0.15 M NaCl, 15 mM Na citrate at pH 7.0). The RNA was transferred to a positively charged nylon membrane (Roche) by vacuum blotting and cross-linked to the membrane in a UV Stratalinker 1800 (Stratagene). The membrane was stained with methlyene blue stain (0.03% methylene blue in 0.3 M sodium acetate at pH 5.2).

Blots were prehybridized for 1 h in Church buffer (0.5 M Na phosphate buffer at pH 6.8, 1 mM EDTA, 7% SDS) at 65°C. ^{32}P-labeled probe was made by amplifying a 695-bp fragment by PCR using rIAN4–690f (5′-CTCCTGGTGGGTAAATCTGG-3′) forward primer and rIAN4–1384r (5′-TCCTTCAGCTCCCT CTTCTG-3′) reverse primer (Invitrogen) in mix containing: 50 ng of genomic DR.*lyp* DNA, 1× TAQ Polymerase Buffer, 250 µM each (dATP, dGTP, dTTP) and 50 µM dCTP, 0.5 µM each primer, 40 µCi ^{32}P-labeled dCTP (PerkinElmer Life Sciences), 0.5 U TAQ 2000 DNA Polymerase (Stratagene). Probe was amplified at 95°C for 3:00 min, then 35 cycles of 95°C for 0:45, 60°C for 0:45, 72°C for 1:00 min.

The probe was purified through a G50 AutoSeq column (Amersham Pharmacia Biotech), denatured by heating at 96°C for 7.5 min, iced, and added to the blot overnight at 65°C. The blot was rinsed twice with 2× SSC/0.1% SDS at room temperature, then washed with 2× SSC/0.1% SDS at 65°C for 20 min, 0.2× SSC/0.1% SDS at 65°C for 20 min, 0.1× SSC/0.1%SDS at 65°C for 30 min. The blot was then placed on BioMax MS Film (Eastman Kodak) and subsequently on a phosphorscreen to be scanned by a STORM 840 PhosphorImager and quantified with `ImageQuant` v1.2 software (Molecular Dynamics).

Blots were stripped by an overnight wash in 0× SSC/0.1% SDS at 65°C and reprobed with rat GAPDH (Accession no. AB017801) cloned into pGEM3z. Using the same method described above, T7 (5′-TAATACGACTCACTATAGGG-3′) forward primer and T3 (5′-ATTAACCCTCACTAAAGGGA-3′) reverse primer were used to generate a 1420-bp fragment in mix containing 1 ng of pGEM3z-rGAPDH, 1× Taq Polymerase Buffer, 250 µM each (dATP, dGTP, dTTP), and 50 µM dCTP, 0.5 µM each primer, 40µCi ^{32}P-labeled dCTP (PerkinElmer Life Sciences), 0.5 U TAQ2000 DNA Polymerase (Stratagene). Probe was amplified at 95°C for 3:00, then 35 cycles of 95°C for 0:45, 50°C for 0:45, 72°C for 1:45.

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Lymphopenia in the BB Rat Model of Type 1 Diabetes is Due to a Mutation in a Novel Immune-Associated Nucleotide (*Ian*)-Related Gene

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