The Diabetes-Prone BB Rat Carries a Frameshift Mutation in *Ian4*, a Positional Candidate of *Iddm1*

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Diabetes-prone (DP) BB rats spontaneously develop insulin-dependent diabetes resembling human type 1 diabetes. They also exhibit lifelong T-cell lymphopenia. Functional and genetic data support the hypothesis that the gene responsible for the lymphopenia, *Lyp*, is also a diabetes susceptibility gene, named *Iddm1*. We constructed a 550-kb P1-derived artificial chromosome contig of the region. Here, we present a corrected genetic map reducing the genetic interval to 0.2 cM and the physical interval to 150–290 kb. A total of 13 genes and six GenomeScan models are assigned to the homologous human DNA segment on HSA7q36.1, 8 of which belong to the family of immune-associated nucleotides (Ian genes). Two of these are orthologous to mouse *Ian1* and *-4*, both excellent candidates for *Iddm1*. In normal rats, they are expressed in the thymus and T-cell regions of the spleen. In the thymus of lymphopenic rats, *Ian1* exhibits wild-type expression patterns, whereas *Ian4* expression is reduced. Mutational screening of their coding sequences revealed a frameshift mutation in *Ian4* among lymphopenic rats. The mutation results in a truncated protein in which the COOH-terminal 215 amino acids—including the anchor localizing the protein to the outer mitochondrial membrane—are replaced by 19 other amino acids. We propose that *Ian4* is identical to *Iddm1*. *Diabetes* 51:1972–1979, 2002

Diabetes in the spontaneously diabetic and lymphopenic BB rat resembles type 1 diabetes in humans. In both diseases, the β-cells are selectively destroyed in an autoimmune process (1). Their lifelong and profound T-cell lymphopenia (2) is characterized by a reduction in $CD4^+$ peripheral T-cells, a severe reduction in $CD8^+$ peripheral T-cells (3), and an almost total absence of $RT6^+$ T-cells (4). The absence of $RT6^+$ T-cells is caused by elevated apoptosis of peripheral T-cells—especially recent thymic migrant cells—and possibly a reduced thymic output of naive T-cells (5–10). This survival defect manifests itself already during the latest stages of intrathymic development (11). In agreement with this, accelerated apoptotic death has been observed among diabetes-prone (DP)-BB mature single-positive thymocytes (12,13), and numbers of $Tcr^{hi}CD4^-8^+$ and $Tcr^{hi}CD4^+8^+$ thymocytes are reduced in the DP-BB rat (6). Taken together, we conclude that the lymphopenia gene product is involved in the regulation of apoptosis in the T-cell lineage and is already expressed during intrathymic development.

Genetically, diabetes and lymphopenia cosegregate in experimental crosses inasmuch as spontaneous diabetes is never observed without lymphopenia (14–16). Several studies have shown that the diabetogenic effect of T-cell lymphopenia is caused by the absence of $RT6^+$ regulatory T-cells (17). $RT6^+$ T-cell depletion of the nonlymphopenic and nondiabetic diabetes-resistant (DR) BB rat induces diabetes in 50% of the animals (18), whereas transfusion of $CD4^+$ T-cells from DR-BB rats to young DP-BB rats reduces the diabetes incidence of recipients in a manner dependent on the number of $RT6^+$ T-cells transferred (19).

The gene responsible for the lymphopenic phenotype (*Iddm1* or *Lyp*) is positioned on rat chromosome 4 (15,20). We have mapped the gene to a 0.3-cM region between markers *D4Got59* and *Abp1* and constructed a 550-kb P1-derived artificial chromosome (PAC) contig covering the region (21). The region is homologous to a segment on human chromosome 7q35–36 (22). In the present study, we refined the genetic interval to a 0.2-cM region between markers *D4Rat214* and *Abp1* corresponding to a physical interval of 150–290 kb. The aim of the present study was to determine which genes are harbored within this interval and ascertain whether any of these fulfill the candidate gene profile and have mutations in lymphopenic rats that could explain the phenotype. Here, we present data to support the hypothesis that the gene *Ian4* fulfills these requirements.

RESEARCH DESIGN AND METHODS

Animals. BB rats (DP-BB and DR-BB) were from the colony kept at the Hagedorn Research Institute (14). Brown Norway (BN), New England Deaconess Hospital (NEDH), and Fischer 344 (F344) rats were obtained from M&B (Lille Skensved, Denmark). Wistar-Furth (WF) rats were obtained from Iffa Credo (Lyon, France).

Genetic mapping. We used a panel of 23 animals known to have recombinations in the vicinity of *Iddm1* (21). The lymphopenic state of the animals was determined from spleen samples using quantitative RT-PCR, which was performed as described below. The primers used for evaluating the *Cd8b* expression level were 5′-TGTCTCAAAGAGCGCCAAGAT-3′ and 5′-CCAG GAACTCAAAGTGCTTGT-3′ (derived from *Cd8b*; GenBank accession no. X04310). Typing of microsatellite markers and *Abp1* was performed as described previously (20–22). Rat *Ian4* was positioned using PCR. The primers 5′-CTGTGACCAGGACCAGTCAGG-3′and 5′-TGGTTCTGGATCTTT GACTCG-3′ gave rise to a 100-bp product from *Ian4*wt and a 99-bp product from *Ian4*bb, as expected. The PCRs were added [^{32}P]-dCTP and run on standard sequencing gels (Gel-Mix 6; Life Technologies, Carlsbad, CA).

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DP, diabetes-prone; DR, diabetes-resistant; PAC, P1-derived artificial chromosome.

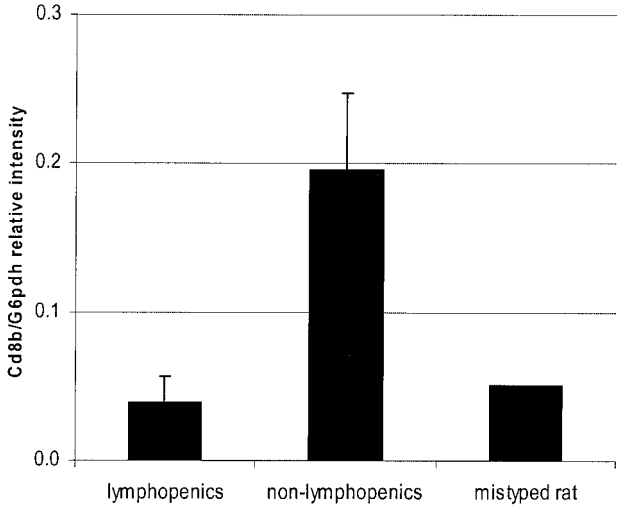

FIG. 1. Lymphopenia-scoring by PCR. Quantitative PCRs were used to evaluate the lymphopenic state of rats. Six lymphopenic and six nonlymphopenic rats from our backcross pedigree were scored (first and second columns). The third column indicates the result from the rat originally mistyped as a nonlymphopenic. Values are the *Cd8b*-specific band intensity divided by the control (*G6pd*) band intensity.

Cloning of *Ian1* and *-4*. PCR primers from human and mouse sequences of *Ian1* and *-4* used to amplify the corresponding rat genes were 5-TGGGGCGT TATACTGTGGAAG-3′ and 5′-ACTCCCGTCTTATCCGTGCTT-3′ (for *Ian1*) and 5′-GTCAAAGGCCCAGAACCAAG-3′ and 5′-AGAGGTCATTGCTGTGGAAGG-3′ (for *Ian4*). 5′- and 3′-RACE were performed using the 5′- and 3′-RACE kits from Life Technologies on thymic RNA from DR-BB rats. Total RNA was isolated from thymocytes with Trizol reagent (Life Technologies). Gene-specific primers used for *Ian1* 5′-RACE were 5′-CTCTCCAGTTCCTCTCTG-3′ and 5′-AGGTTTGTT TCTGAATCACAC-3′. The gene-specific primer used for *Ian1* 3′-RACE was 5′-GAGACGGCTCCTGAAGTCCTT-3′. Gene-specific primers used for *Ian4* 5′-RACE were 5′-TTCTCACCAGGGCCATGA-3′, 5′-GGGTGAAGAGGACAATCAT GT-3′, and 5′-AGGTAGCAGTTCCCAATGTCC-3′. The gene-specific primer used for *Ian4* 3′-RACE was 5′-GGACATTGGGAACTGCTACCT-3′. PCR products were cloned in pCR2.1-TOPO or pCRII-TOPO with a TOPO TA cloning kit (Invitrogen, Gronningen, the Netherlands).

Sequencing and sequence analysis. Overlapping fragments of 300–500 bp from *Ian1* and *-4* were PCR-amplified, and PCR products were cloned for sequencing of the coding sequences of the genes. For *Ian1* the following primer pairs were used: 5′-CCCAAATTTTCAGGGATCTGA-3′/5′-ACCAGAAG CAGAGCATGAGGC-3′, 5′-AGATTACTCGCTGTGTTGCTC-3′/5′-TCTGCTTC ATACTCCCGTCTT-3, and 5′-AAGTGCTGAGGGTGTGATTCA-3′/5′-TTGGCCA TGGTGTTTGTTATG-3′. For *Ian4* the following primer pairs were used: 5′-TTTTTCCTGCTTCGGACTCAG-3′/5′-GCCTGGACTCGAACGCTGGTC-3′ (specific for *Ian4S*), 5′-TTATAGCCAAACAGTTAGGAG-3′/5′-TTTCCGCAGCC AGATTTACCC-3′ (specific for *Ian4 L*), 5′-GGATCCTCCTGGTGGGTAAAT-3′/ 5′-GGGTGAAGAGGACAATCATGT-3′, 5′-AACTGGGACGCTACACAGTCG-3′/ 5′-TTCACTGTAGCCACCTCTAAG-3′, and 5′-GGCCCTGGTGAGAAGACTGG A-3′/5′-GGCAGGATTCACCCAAAGAGA-3′. At least two independently cloned PCR products from each primer set were sequenced to avoid PCR artifacts. Sequencing was performed by MWG-Biotech. Protein sequences were aligned using BLASTp (available online at www.ncbi.nlm.nih.gov/BLAST), followed by fine adjustment using visual inspection. Transmembrane regions were defined in silico using the TMHMM server (available online at www.cbs.dtu.dk/services/TMHMM-2.0).

Expression profiling. Tissue-specific expression patterns were determined by PCR using Rat Multiple Choice cDNAs (OriGene Technologies). Quantitative differences in expression levels in thymus were determined using quantitative RT-PCR on cDNA made with SuperScript (Life Technologies) from DR- and DP-BB total thymic RNA (made with TRIZOL Reagent; Life Technologies). [^{32}P]-dCTP was added to the PCRs, which were then run on standard sequencing gels (Gel-Mix 6; Life Technologies), and primer-specific bands were quantitated using a Typhoon 8600 Variable Mode Imager (Amersham-Pharmacia Biotech, Little Chalfont, U.K.). Primers specific for *Ian1* and *-4* are described above. Primers specific for the housekeeping gene *G6pd* (5′-GACCTGCAGAGCTCCAATCAAC-3′ and 5′-CACGACCCTCAGTACCAA AGGG-3′ from GenBank accession no. NM_017006) were used as an internal control in all reactions.

In situ hybridization. In situ hybridization was performed on paraffin sections that were heated to 60°C for 30 min, deparaffinized in xylene, and rehydrated. The slides were acid-treated in 0.2 mol/l HCl, treated with proteinase K (5 μg/ml), and subsequently fixed in 4% paraformaldehyde, followed by dehydration before the ^{35}S-UTP–labeled antisense or sense probes were added. The sections were then incubated overnight at 47°C in a hybridization solution containing radiolabeled RNA probe (80 pg/μl) in a solution of 50% deionized formamide, 10% dextran sulfate, tRNA (1 μg/ml), ficoll 400 (0.02% wt/vol), 0.02% polyvinylpyrrolidone (wt/vol), 0.2% BSA fraction V (wt/vol), 10 mmol/l dithiothreitol, 0.3 mol/l NaCl, 0.5 mmol/l EDTA, 10 mmol/l Tris-Cl, and 10 mmol/l sodium phosphate (pH 6.8). After hybridization, slides were washed at 57 and 62°C. The sections were then treated with RNase A (20 μg/ml), dehydrated, and air-dried. Autoradiographic film and subsequent emulsion was applied, and the sections were developed after 2–3 weeks of exposure. The ^{35}S-UTP–labeled probe was made from the same rat-specific PCR products used for RT-PCR cloned in the pCRII-TOPO vector (see above). Antisense probe was made by in vitro transcription using the SP6 promoter, whereas sense (control) probe was made by in vitro transcription of the same plasmid from the T7 promoter. All probe preparations, including both sense and antisense probes, were adjusted to 2×10^6 cpm/μl. Probes were stored at −20°C until use.

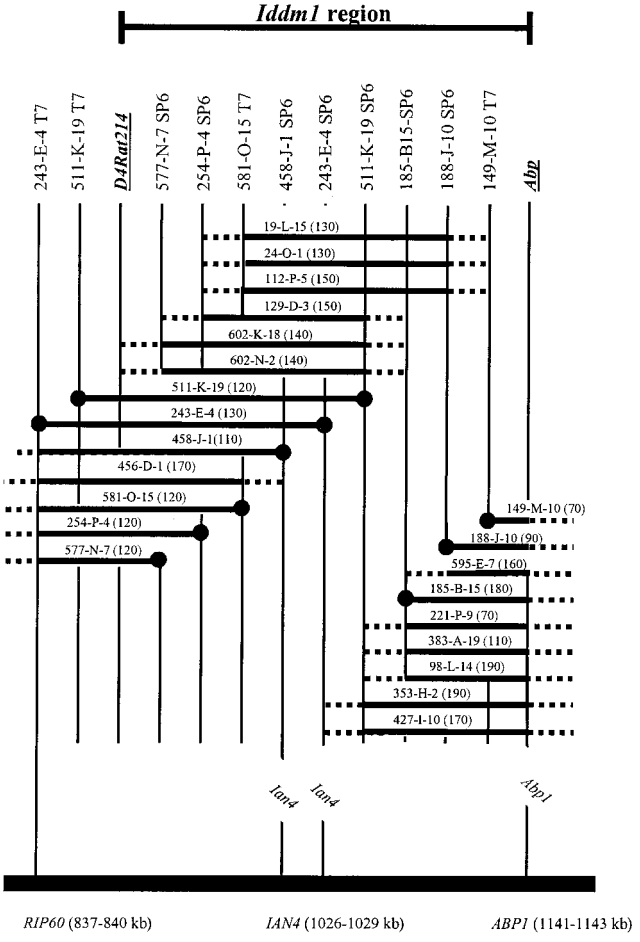

FIG. 2. Map of the PAC contig covering the Lyp region. Markers used for the contig construction are given equidistantly on top. Underlined markers were used for genetic mapping and were used for screening the entire PAC library (21). Horizontal lines represent PACs from the RCPI-31 rat PAC library (ResGen, Huntsville, AL). Dashed ends indicate that the precise end points are unknown. ●, the origin of a marker from the end of a PAC. The official (commercially available) PAC names are listed above each PAC, with sizes in kilobases in parentheses. The gene names written diagonally below the contig are mouse genes homologous to the indicated PAC ends. The black bar on the bottom represents the homologous human chromosome 7 draft segment (GenBank accession no. NT_007704). Lines extended to the human segment indicate homology, and the name of the genes and their positions in the segment are given below.

TABLE 1
Genes between *RIP60* and *ABP1* on HSA7q36.1

	Location†	Best BLASTp hit to known protein
Gene*		
LOC136359 (XM_059840)	835–864	Zinc finger protein 41 (XP_033888)
LOC136361 (XM_072457)	866–878	No hits
LOC136362 (XM_072461)	874–876	No hits
LOC90512 (XM_032237)	978–980	*ALS2CR2* (NP_061041)
FLJ11110 (NM_018326)	1033–1040	Mouse *Ian1* (no GenBank file); probable orthologue
LOC136363 (XM_069849)	1079–1080	Rat transient receptor protein 6 (NP_446011)
FLJ22690 (NM_024711)	1091–1098	Mouse *Ian3* (no GenBank file) and Mouse *Ian4* (AAK31138)
LOC115261 (XM_032229)	1151–1159	Mouse *Ian4* (AAK31138)
LOC136364 (AL080068)	1203–1205	No open reading frame
FLJ11296 (NM_018384)	1026–1028	Mouse *Ian4* (AAK31138); probable orthologue
LOC136365 (XM_069850)	1213–1245	Mouse *Ian4* (AAK31138)
LR8 (NM_014020)	1257–1267	—
LOC115262 (XM_004745)	1267–1271	*HCA112* (NM_018487)
GenomeScan model		
Hs7_7861_26_7_2	871–874	Mouse zinc finger protein 29 (NP_033579)
Hs7_7861_26_7_3	894–944	Mouse *Ian1* (no GenBank file), Mouse *Ian3* (no GenBank file) and Mouse *Ian4* (AAK31138)
Hs7_7861_26_9_1	986–1004	Mouse *Ian1* (no GenBank file)
Hs7_7861_27_13_1	1108–1187	Mouse *Ian4* (AAK31138)
Hs7_7861_27_13_2	1200–1203	No hits
Hs7_7861_27_18_1	1280–1302	No hits

*GenBank sequences are given in parentheses; †location is the kilobase position in HSA7 contig NT_007704.

RESULTS

Refined mapping of Iddm1 to a 0.2-cM region. Our previous genetic mapping of *Iddm1* was dependent on a few recombinations, each influencing the fine positioning of the gene within the region between *D4Got59* and *Abp1* (22). Indeed, only three rats from our experimental crosses between the DP-BB rat and three different strains of nonlymphopenic rats exhibited recombinations within this interval. Phenotyping of their lymphopenic state (i.e., homozygosity versus heterozygosity for the mutated *Iddm1*) had been performed on blood samples taken from live animals by flowcytometric evaluations of the frequency of $TCR_{\alpha\beta}$-labeled cells among white blood cells (described in 16), whereas the genotyping of microsatellite markers (and *Abp1*) was based on the PCR of genomic DNA from organ samples procured at the time of killing. This procedure cannot rule out the possibility of a sample mismatch. We therefore decided to verify the lymphopenic phenotype and marker genotyping on the same frozen samples. For lymphopenia detection, we chose to perform quantitative RT-PCR for *Cd8b* levels in the spleen because the number of CD8-positive T-cells is severely reduced in lymphopenic rats (3). Spleen samples from the panel of backcross animals harboring recombinations in the region (including the three aforementioned animals) were thus used. All previous data were verified, except for one case where the quantitative RT-PCR unambiguously typed the splenic sample as lymphopenic, although our previous records of blood samples had typed it as nonlymphopenic (Fig. 1). This results in a refined position of *Iddm1* to the 0.2-cM interval between markers *D4Rat214* and *Abp1*, corresponding to a maximum of 290 kb (Fig. 2). This position relative to any other is supported by a log-likelihood of 5.3.

A gene map of the Iddm1-region. As indicated in Fig. 2, four of the rat PAC ends exhibit homology to mouse and human sequences. This enabled us to align the rat *Iddm1* region with a region on human chromosome 7 that has been completely sequenced (GenBank accession no. NT_007704). A total of 13 genes and six GenomeScan models have been assigned a position between *RIP60* and *ABP1* on human chromosome 7 according to the National Center for Biotechnology Information (NCBI) *Homo sapiens* map view of HSA7 (available online at www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search) (Table 1). As can be seen from the Fig. 2, this region encompasses the entire homologous *Iddm1* region in the rat.

Because only one of the genes has been characterized (LR8; a gene expressed by lung fibroblasts) (23), we searched the GenBank for similarity to known genes. Five genes (LOC136361, LOC136362, LOC136364, Hs7_7861_27_13_2, and Hs7_7861_27_18_1) exhibit no similarity to any known gene. The protein encoded by LOC136359 exhibits low similarity to human zinc finger protein 41 (24). LOC90512 is 89% identical to the protein encoded by *ALS2CR2*, a probable protein kinase (25). The protein encoded by LOC136363 is similar to the COOH-terminal end of rat transient receptor protein 6, a Ca transport protein encoded by *Trrp6* (26). The protein encoded by LOC115262 is 99% identical to the protein encoded by *HCA112* (locus link no. 55365), a gene with unknown function, but some similarity to *LR8* (23). The GenomeScan model gene Hs7_7861_26_7_2 exhibits similarity to mouse zinc finger protein 29 (27). The remaining eight genes all exhibit similarity to members of a family of GTP-binding proteins, the immune-associated nucleotides (Ian genes). Three members of this gene family have been described: *Ian1* is a thymic selection marker expressed at various stages of thymocyte development (28). *Ian2* is induced in B-lymphocytes by infections of *Plasmodium chabaudi* malaria (29,30). *Ian3* (28) has not been functionally characterized. *Ian4* encodes a GTP-binding protein localized in the outer mitochondrial membrane, and it is

expressed in both spleen and a mouse T-cell line (31). The gene is overexpressed in hematopoietic precursor 32D cells transfected with a Bcr/Abl oncogene, which is involved in several leukemias.

Another gene with high similarity to the *Ian* genes is the *Arabidopsis AIG1* gene, which is induced during a bacterial resistance response leading to apoptosis (37). The orthologues of both mouse *Ian1* and *Ian4* seem to be included in our interval: FLJ11110 encodes a 329–amino acid protein that exhibits 61% identity to the protein encoded by mouse *Ian1* (a 328–amino acid protein), whereas FLJ11296 encodes a 307–amino acid protein that exhibits 61% identity to the protein encoded by mouse *Ian4* (a 295–amino acid protein). The remaining seven genes exhibit a lower similarity to either of the two mouse genes. The *Ian* genes seem to be predominantly expressed in lymphoid tissues, and a role in apoptosis can be inferred by the function of *AIG1*. Therefore, we believed these genes to be good candidates for *Iddm1*. Because the functions of *Ian1* and *-4* make them obvious candidates, we decided to characterize them.

Cloning of rat *Ian1* and *-4*. We designed primers specific for the rat orthologue of *Ian1* and *Ian4* by placing primers in a region of homology between human and mouse genes. The resulting PCR products were sequenced, and the sequence was used to isolate the entire coding region using the rapid amplification of cDNA end (RACE) procedure. For rat *Ian1*, a 1,423-bp transcript was discovered (GenBank accession no. AY070268). An open reading frame of 310 bp was observed. The alignment of rat, mouse, and human sequences is shown in Fig. 3*A*. For rat *Ian4*, two different 5′ ends were discovered, resulting in transcripts of 1,245 bp and 1,310 bp, respectively. The two transcripts contain two open reading frames of, respectively, 334 and 308 codons (Ian4 L and Ian4S; GenBank accession nos. AY055776 and AY055777), the last 293 codons being identical. Both are highly homologous to both mouse Ian4 and to human IAN4 proteins. The alignment of rat, mouse, and human sequences shown in Fig. 3*B* suggests that *IAN4* is indeed the human orthologue of murine *Ian4*, albeit Dahéron et al. (31) named the human gene *IAN5*. Although the sequences differ in their COOH-terminal ends, they all contain a putative transmembrane tail.

Expression pattern of *Ian1* and *-4*. Using a panel of tissue-specific cDNAs, we determined the expression patterns of *Ian1* and *-4*. *Ian1* is primarily expressed in spleen, thymus, heart, lung, and intestine, and it is expressed, to a lesser degree, in liver, kidney, stomach, and muscle. *Ian4* (both isoforms*)* is primarily expressed in spleen, thymus, heart, lung, and intestine, and it is expressed, to a lesser degree, in kidney, stomach, and muscle (Fig. 4). We then performed in situ hybridization of the two genes on thymus and spleen. In the DR-BB rat, *Ian1* mRNA is expressed in the periarteriolar lymphatic sheets of the spleen (Fig. 5*Aa*, *Ae*, and *Af*) and in thymic medulla (Fig. 5*Ba*, *Be*, and *Bf*). In the DP-BB rat, *Ian1* seems to be expressed at reduced levels in both spleen and thymus (Fig. 5*Ac*, *Ag*, *Ah*, *Bc*, *Bg*, and *Bh*). For *Ian4*, the DR-BB expression pattern is as follows: in the spleen, *Ian4* is expressed primarily in the periarteriolar lymphatic sheets (Fig. 6*Aa*, *Ae*, and *Af*), whereas in the thymus, *Ian4* mRNA

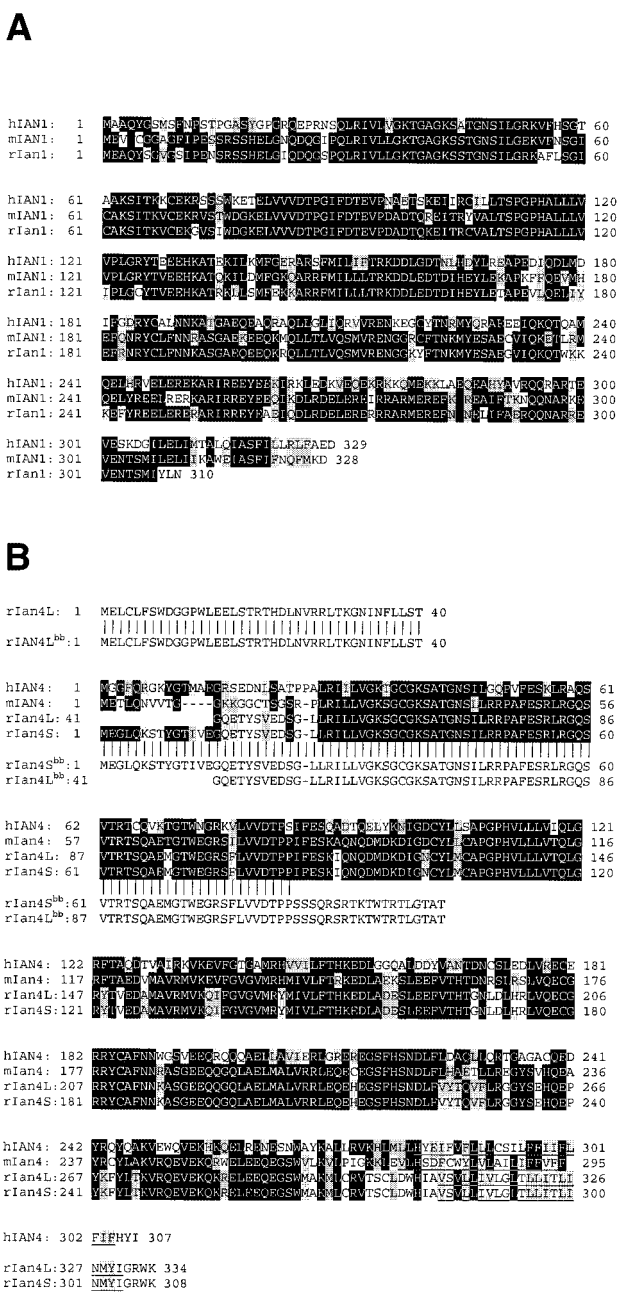

FIG. 3. *A*: Alignment of protein sequences of Ian1 of human (hIAN1), mouse (mIan1), and rat (rIan1) origin. Dark shading indicates identical amino acids, whereas light shading indicates conserved amino acids. No transmembrane regions were detected. *B*: Protein sequences of Ian4 of human (hIAN4), mouse (mIan4), and rat (rIan4S and rIan4 L) origin aligned with the DP-BB rat alleles (rIan4S^{bb} and rIan4L^{bb}). Dark shading indicates identical amino acids, whereas light shading indicates conserved amino acids. Predicted transmembrane regions are underlined. The missense mutations are all positioned after the new stop-codon introduced by the frameshift mutation, and they therefore have no effect on the Ian4^{bb} gene products.

is evenly distributed all over the thymus (Fig. 6*Ba*, *Be*, and *Bf*). In the DP-BB rat, very low amounts of *Ian4* are expressed in both spleen and thymus (Fig. 6*Ac*, *Ag*, *Ah*, *Bc*, *Bg*, and *Bh*). Because expression level differences in the thymus might be caused by a promoter mutation in the gene (that might be the primary gene defect leading to lymphopenia), differences in thymic expression levels were also evaluated by quantitative RT-PCR on thymus cDNA of DP- and DR-BB origin. No significant difference

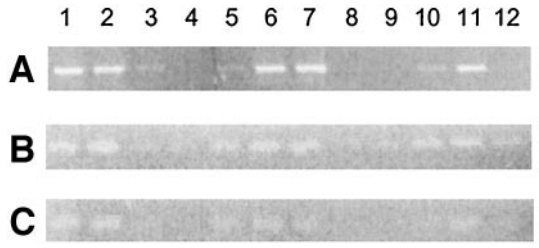

FIG. 4. Tissue-specific expression pattern of Ian1 and -4. PCR analysis on tissue-specific cDNA pools for *Ian1* (*A*), *Ian4 l* (*B*), and *Ian4S* (*C*). The lanes are: *lane 1*, spleen; *lane 2*, thymus; *lane 3*, liver; *lane 4*, brain; *lane 5*, kidney; *lane 6*, heart; *lane 7*, lung; *lane 8*, testis; *lane 9*, skin; *lane 10*, stomach; *lane 11*, small intestine; *lane 12*, muscle.

between DP- and DR-BB rat levels was found for *Ian1*, whereas for *Ian4* we detected a 3.4-fold higher thymic expression level in the DR-BB rat relative to the DP-BB rat (Fig. 7).

A frameshift mutation in the BB allele of the *Ian4* gene leads to a truncated protein. The coding region of *Ian1* and *-4* were screened for mutations in the DP-BB rat relative to the nonlymphopenic DR-BB rat. For *Ian1*, two allele differences were detected: one silent and one conservative (Ala55Val) mutation. For *Ian4*, five allele differences were detected: two silent mutations, two conservative missense mutations (Ala156Val and Met203Ile), and one frameshift mutation (450delC). The frameshift mutation results in a mutated DP-BB allele ($Ian4^{bb}$) encoding a truncated protein, where the 215 last amino acids of wild-type *Ian4* ($Ian4^{wt}$) are replaced by 19 other amino acids (Fig. 3*B*). The COOH-terminal transmembrane region is missing altogether, and since the 20-most COOH-terminal amino acids of mouse *Ian4* are necessary for localization of the gene product in the outer mitochondrial membrane (31), the $Ian4^{bb}$ gene product is probably not correctly localized. The $Ian4^{wt}$ sequence was confirmed in four other nonlymphopenic rat strains (BN, WF, NEDH, and F344), whereas the $Ian4^{bb}$ sequence was confirmed in two other lymphopenic rat strains, namely a WF rat congenic for the lymphopenia locus of DP-BB origin and a F344 rat congenic for the lymphopenia locus and the major histocompatibility complex of DP-BB origin (32–33). The single-nucleotide deletion in $Ian4^{bb}$ gives rise to a polymorphism that we used for genetic mapping of *Ian4*. We positioned *Ian4* to the same position as *Iddm1*, between *D4Rat214* and *Abp1* (data not shown).

DISCUSSION

We have refined the genetic positioning of rat *Iddm1* to a 0.2-cM (or 150- to 290-bp) region on rat chromosome 4 and

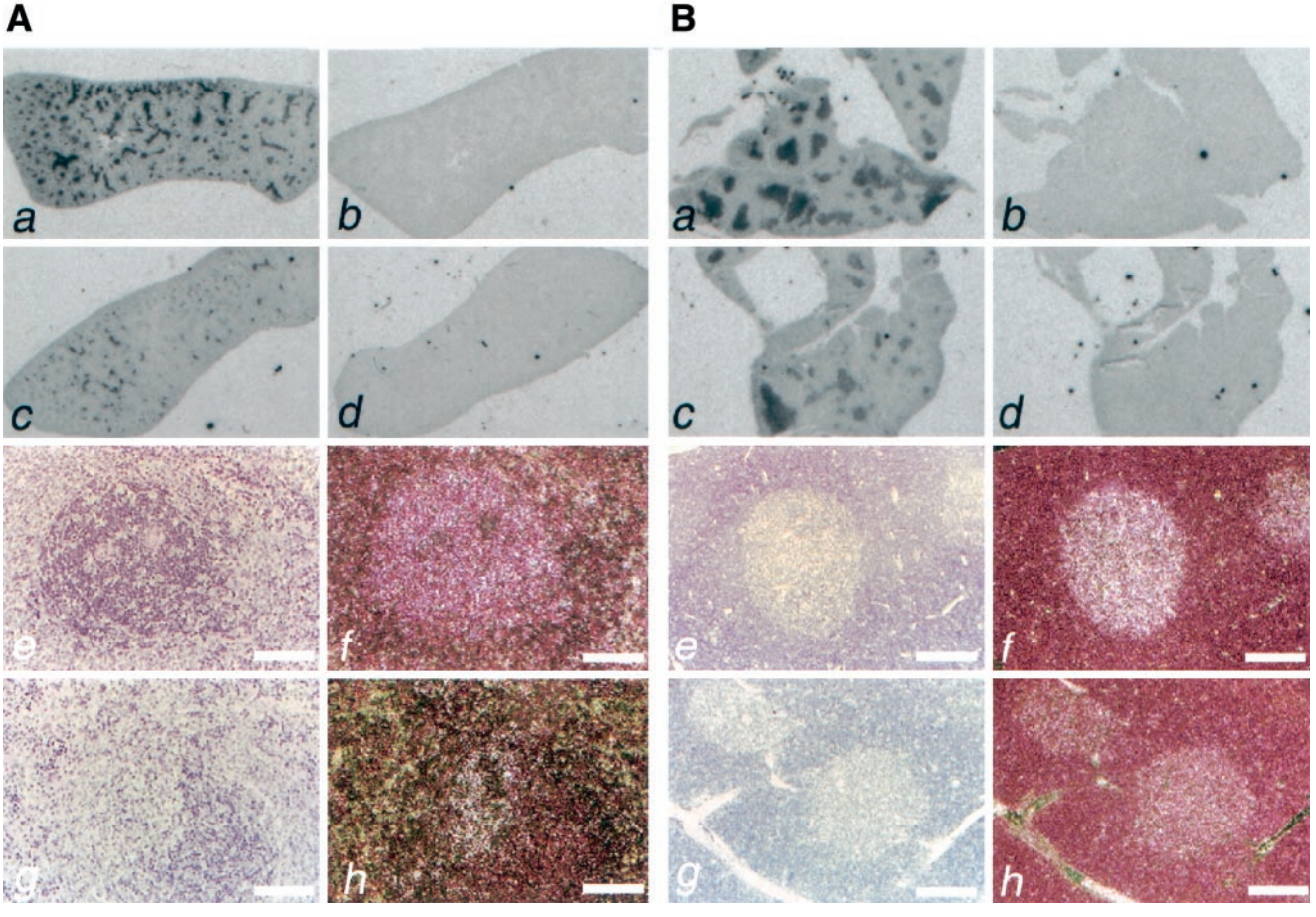

FIG. 5. Expression analysis of *Ian1*. *A*: Spleen. *Ian1* mRNA is expressed in the spleen in the periarteriolar lymphatic sheets in DR-BB rats (*a*, *e*, and *f*) and in lower amounts in DP-BB rats (*c*, *g*, and *h*) as revealed by in situ hybridization with an *Ian1* antisense probe. The corresponding *Ian1* sense probe gave no specific hybridization signal (*b* and *d*). Panels *f* and *h* are dark-field images of the same areas as shown in *e* and *g*. *B*: Thymus. *Ian1* mRNA is expressed in the medulla in both DR-BB rats (*a*, *e*, and *f*) and in DP-BB rats (*c*, *g*, and *h*), as revealed by in situ hybridization with an Ian4 antisense probe. The corresponding *Ian1* sense probe gave no specific hybridization signal (*b* and *d*). Panels *f* and *h* are dark-field images of the same areas as shown in *e* and *g*. Bars: 100 μm.

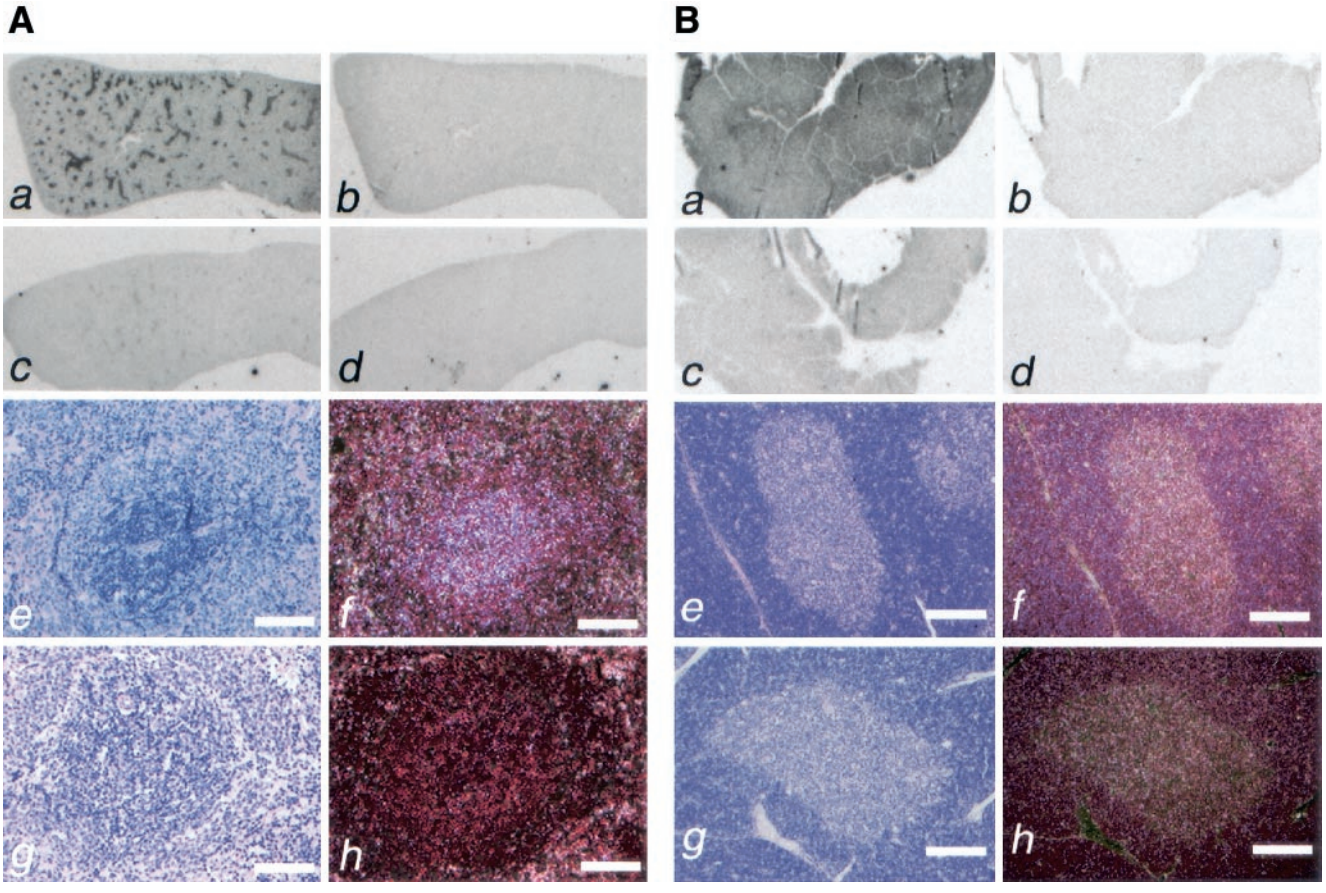

FIG. 6. Expression analysis of *Ian4*. *A*: Spleen. *Ian4* mRNA is expressed in the spleen in the periarteriolar lymphatic sheets in DR-BB rats (*a*, *e*, and *f*) and in lower amounts in DP-BB rats (*c*, *g*, and *h*) as revealed by in situ hybridization with an *Ian4* antisense probe. The corresponding *Ian4* sense probe gave no specific hybridization signal (*b* and *d*). Panels *f* and *h* are dark-field images of the same areas as shown in *e* and *g*. *B*: Thymus. *Ian4* mRNA is expressed uniformly all over the thymus in DR-BB rats (*a*, *e*, and *f*) and much lower in DP-BB rats (*c*, *g*, and *h*) as revealed by in situ hybridization with an *Ian4* antisense probe. The corresponding *Ian4* sense probe gave no specific hybridization signal (*b* and *d*). Panels *f* and *h* are dark-field images of the same areas as shown in *e* and *g*. Bars: 100 μm.

produced a transcript map of this region by comparative mapping to humans. Two candidate genes were chosen, and the rat homologues were cloned. Both candidate genes were subjected to expression profiling, which showed both of them to be expressed in the thymus, where *Iddm1* is expected to be expressed. Dahéron et al. (31) detected low levels of mouse *Ian4* in the spleen, but not in the thymus. This discrepancy between our data and theirs might be due to differences between rat and mouse expression patterns and perhaps the differently applied techniques, since Dahéron et al. used a Northern blotting technique. However, we confirmed our observations using in situ hybridizations. The confinement to the periarteriolar lymphatic sheets in the spleen confirms a role of both genes in the T-cell lineage, as suggested already by the expression in the thymus. The in situ hybridizations showed *Ian4* to be expressed all over the thymus, suggesting that the expression is important at all time points during thymic development. In contrast, *Ian1* is only expressed in the thymic medulla, where the most mature thymocytes reside (34). The in situ hybridizations also suggested a reduced expression level of the two genes in both thymus and spleen. Because of the T-cell lymphopenia in the DP-BB rat, a reduced expression in spleen is expected for all T-cell–specific transcripts in the spleen, whereas a reduced thymic expression should only be expected from a subset of such genes, including *Iddm1* itself.

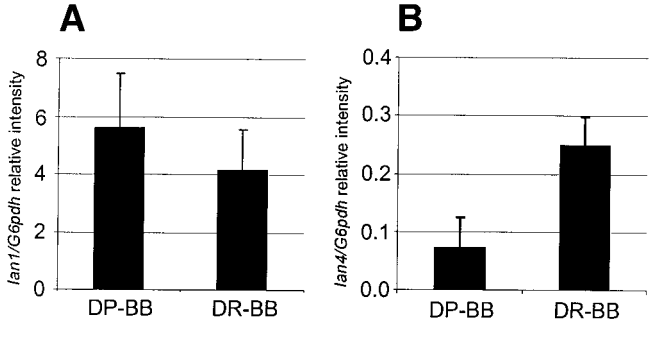

FIG. 7. Quantitative RT-PCR of rat *Ian1* and *-4* in rat thymus. Five DP-BB and five DR-BB rats were scored for *Ian1* (*A*) and *Ian4* (*B*). Values are the Ian-specific band intensity divided by the control (*G6pdh*) band intensity.

Quantitative RT-PCR confirmed the lower expression of *Ian4*—but not *Ian1*—among DP-BB thymocytes as compared with DR-BB thymocytes. This could be caused by a secondary effect of the lymphopenia or a mutation in *Ian4* itself, either as a result of a promoter mutation or as a result of nonsense-mediated mRNA decay, which is seen as a result of both nonsense and frameshift mutations (35). We screened the coding sequences of the genes by RT-PCR

and found a frameshift mutation in *Ian4*. This mutation results in a truncated gene product that lacks the 205-most COOH-terminal codons (or two-thirds of the total protein), including the COOH-terminal transmembrane region sequence that localizes *Ian4* to the outer mitochondrial membrane in the mouse. Apart from this mutation, only silent or conservative mutations were discovered in either gene.

Considering the impact of the frameshift mutation on the protein, it is unlikely that the $Ian4^{bb}$ gene product is able to maintain the function of the wild-type gene product. We have thus detected a deleterious mutation in a gene present in the narrow *Iddm1* region. Several observations support the hypothesis that *Ian4* is involved in the regulation of T-cell apoptosis, making it an excellent candidate for *Iddm1*. First, high expression of *Ian4* is associated with leukemia in the mouse (31), whereas absent/low expression appears to be associated with lymphopenia. Second, the gene product is anchored to the outer mitochondrial membrane, and this organelle is an important site for the regulation of cell death (36). Third, the highly homologous *Ian1* gene is differentially expressed during thymocyte development, a process known to involve apoptotic events, and the related plant gene, *AIG1*, is involved in hypersensitivity response against bacterial pathogens, a process leading to apoptosis (37), both suggesting the involvement of Ian family members in the control of apoptosis. Finally, *Ian4* is a GTP-binding protein, and small GTP-binding proteins such as *Drosophilae* Ras and rice Rac have been shown to be involved in apoptosis (36). In addition, our data show that *Ian4* is expressed both in thymus and spleen, and that it is confined to the T-cell–rich areas of the latter. Altogether, this suggests that *Ian4* protects against apoptosis among T-cells. We believe the circumstantial evidence presented here establishes *Ian4* as being identical to rat *Iddm1*.

Whether the human homologue of rat *Iddm1* plays a role in human type 1 diabetes is unknown, but we have performed an association study using a closely positioned microsatellite marker on a Danish patient population (38). This study did not show any association to human type 1 diabetes. Although the marker was positioned only 14 kb from human *IAN4*, we cannot, however, rule out an association with the gene itself. Moreover, other genes involved in the generation of regulatory T-cells may be type 1 diabetes susceptibility genes in humans, as is indeed *CTLA4* (39). Under all circumstances, the identification of rat *Iddm1* is of importance for the elucidation of the precise mechanisms for T-cell survival and the generation of regulatory T-cells.

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