Original Article

Introgression of F344 Rat Genomic DNA on BB Rat Chromosome 4 Generates Diabetes-Resistant Lymphopenic BB Rats

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Failure to express the *Gimap5* protein is associated with lymphopenia (*lyp*) and linked to spontaneous diabetes in the diabetes-prone BioBreeding (BBDP) rat. *Gimap5* is a member of seven related genes located within 150 Kb on rat chromosome 4. Congenic DR.$^{lyp/lyp}$ rats, where BBDP *lyp* was introgressed onto the diabetes-resistant BBDR background (BBDR.BBDP.$^{lyp/lyp}$), all develop diabetes between 46 and 81 days of age (mean ± SE, 61 ± 1), whereas DR.$^{lyp/+}$ and DR.$^{+/+}$ rats are nonlymphopenic and diabetes resistant. In an intercross between F1(BBDP x F344) rats, we identified a rat with a recombination event on chromosome 4, allowing us to fix 33 Mb of F344 between D4Rat253 and D4Rhw6 in the congenic DR.*lyp* rat line. *Gimap1* and *Gimap5* were the only members of the *Gimap* family remaining homozygous for the BBDP allele. Offspring homozygous for the F344 allele (*f/f*) between D4Rat253 and D4Rhw6 were lymphopenic (85 of 85, 100%) but did not develop diabetes (0 of 85). During rescue of the recombination, 102 of 163 (63%) rats heterozygous (*b/f*) for the recombination developed diabetes between 52 and 222 days of age (88 ± 3). Our data demonstrate that introgression of a 33-Mb region of the F344 genome, proximal to the mutated *Gimap5* gene, renders the rat diabetes resistant despite being lymphopenic. Spontaneous diabetes in the BB rat may therefore be controlled, in part, by a diabetogenic factor(s), perhaps unrelated to the *Gimap5* mutation on rat chromosome 4. *Diabetes* 55:3351–3357, 2006

The BioBreeding (BB) rat shares characteristics common to human type 1 diabetes including polyuria, hyperglycemia, ketoacidosis, insulitis, and insulin dependency for life. As in human type 1 diabetes, islets are infiltrated by mononuclear cells at the time of clinical onset, and insulitis is associated with a rapid onset of hyperglycemia due to a complete loss of islet β-cells (rev. in 1). In human type 1 diabetes, a number of critical steps in the disease process need to be clarified. For example, the genetic etiology is not fully understood. While HLA is the major susceptibility factor, other genetic factors or loci have been identified through linkage (sib-pair) (2) or association (case-control) (3,4) approaches. In addition, whether environmental etiological factors, such as viruses, are able to trigger the islet autoimmunity that precedes clinical diagnosis is still a matter of debate (5,6). In contrast, however, BioBreeding (BB) rat type 1 diabetes appears to be controlled by a limited number of genetic factors in inbred congenic BBDR.BBDP-(D4Mit6-D4Mit7)/Rhw (DR.*lyp*) rats housed under specific pathogen–free conditions (7–9). The first major susceptibility factor, major histocompatibility complex (MHC) haplotype RT1.B$^{u/u}$ (*Iddm1*), an ortholog of HLA, is located on chromosome 20 (10,11). The second major susceptibility factor, lymphopenia (*lyp;Iddm2*) is caused by a frameshift mutation in the *Gimap5* gene on chromosome 4 (12,13). *Gimap5* belongs to a family of at least seven GTPase immune-associated protein (*Gimap*) encoding genes (14), all of which are differentially expressed in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ T- and B-lymphocytes, as well as macrophages and dendritic cells (12,13,15,16). Genome-wide linkage analysis for the diabetes phenotype have identified additional loci after crossing different inbred BB rats with DA rats (*Iddm3* on chromosome 18) (17), WF rats (*Iddm4* and *Iddm5* on chromosomes 4 and 13, respectively) (18,19), and SHR rats (*Iddm6* on the X chromosome) (20). These genetic factors therefore seem to be dependent on the rat strain used in the cross, i.e., influenced by a differing genome background.

Our approach to dissect the genetics of spontaneous diabetes in the BB rat was to initially develop lines congenic for *lyp* through introgression of the BBDP *lyp* locus onto BBDR to generate DR.$^{lyp/lyp}$ rats (7). Characterization of this congenic strain confirmed the original observation that diabetes segregates with lymphopenia (21). Next, in a cross-intercross between BBDP and Lewis rats, we confirmed that the MHC locus on chromosome 20 was linked to diabetes (22). Additionally, in a cross between DR.$^{lyp/lyp}$ and F344 rats, we found evidence for a third factor in F344 that conferred resistance to diabetes (22). This resistance was eventually dissected into two loci, one conferring ~60% protection on chromosome 2 (*Iddm3*) (8) and the another conferring ~40% protection on chromosome 15 (*Iddm19*) (A.E.K., S. Speros, M.

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Received for publication 24 May 2006 and accepted in revised form 31 August 2006.

MHC, major histocompatibility complex; SSLP, simple sequence–length polymorphism; STS, sequence-tagged site.

DOI: 10.2337/db06-0715

Marker	Mb	BBDP	BBDR	F344	1	2	3	4	5	6	7	8
Chr04												
D4Rat139	3.04	137	143	141	4	1	2	2	5	2	2	2
D4Rat153	42.53	150	138	150	4	1	2	2	5	2	2	2
D4Rat253	44.55	151	165	165	4	1	2	2	5	2	6	4
D4Arb11	59.47	237	237	227	4	1	2	2	5	2	6	4
D4Rat102	66.22	257	257	237	4	1	2	2	5	2	6	4
D4Mgh32	69.09	392	392	410	4	1	2	2	5	2	6	4
D4Rat26	69.18	167	167	157	4	1	2	2	5	2	6	4
D4Rat19	70.64	134	140	142	4	1	2	2	5	2	6	4
D4Rat163	72.70	249	251	267	4	1	2	2	5	2	6	4
D4Rat164	73.01	183	195	175	4	1	2	2	5	2	6	4
D4Mgh24	73.75	141	133	149	4	1	2	2	5	2	6	4
D4Rhw11	75.81	223	218	235	4	1	2	2	5	2	6	4
D4Rhw12	76.07	230	240	247	4	1	2	1	5	1	5	4
Gimap8	76.74				4	1	2	1	5	1	5	4
Gimap9	76.77				4	1	2	1	5	1	5	4
D4Rhw2	76.77	175	180	178	4	1	2	1	5	1	5	4
Gimap4	76.78				4	1	2	1	5	1	5	4
Gimap6	76.79				4	1	2	1	5	1	5	4
Gimap7	76.81				4	1	2	1	5	1	5	4
D4Rhw6	76.83	210	200	205	4	1	2	1	5	1	5	4
Gimap1	76.83				4	1	2	1	1	1	1	1
Gimap5	76.84				4	1	2	1	1	1	1	1
D4Rhw8	76.84	175	180	178	4	1	2	1	1	1	1	1
D4Rhw13	77.41	172	160	168	4	1	2	1	1	1	1	1
D4Rhw10	77.81	286	297	290	4	1	2	2	1	2	3	1
D4Mit24	78.04	134	136	140	4	1	2	2	1	2	3	1
D4Rat77	95.50	161	165	155	4	1	2	2	1	2	3	1
D4Rat39	104.19	158	150	156	4	1	2	2	1	2	3	1
D4Rat61	153.36	169	162	160	4	1	2	2	1	2	3	1
D4Rat62	157.91	168	164	162	4	1	2	2	1	2	3	1

FIG. 1. Genotyping of chromosome 4. STS/SSLP marker map of chromosome 4. Marker annotations are shown together with the University of California Santa Cruz map marker location and the size of the marker in the three parental strains BBDP, BBDR, and F344. Light blue indicates markers polymorphic between all three strains. The animals in *lanes 1–8* represent the parental and new DRF congenic lines. The animal in *lane 1* is F344, *lane 2* is BBDP, *lane 3* is BBDR, *lane 4* is DR.*lyp/lyp*, *lane 5* is the original recombinant rat, *lane 6* is DRF.*b/b*, *lane 7* is DRF.*b/ff*, and *lane 8* is DRF.*ff*. The numerical color code is as follows: yellow (1) DP/DP, blue (2) DR/DR, green (3) DP/DR, red (4) F344/F344, orange (5) DP/F344, and purple (6) DR/F344.

Tschannen, J.M.F., A.J.M., B. Majewski, H. Jacob, A.L., unpublished observations). In identifying these resistance factors, we relied heavily on generating F2(BBDP X F344) rats to maximize recombination events within the genome. One rat, previously reported (12) (Fig. 1), showed a recombination event within the *lyp* critical interval that left *Gimap5* and *Gimap1* as DP and the remaining *Gimap* genes, contained in a 33-Mb fragment, as F344. The aim of this study was to secure introgression of the 33-Mb F344 fragment onto the congenic DR.*lyp* rat line through a series of marker-assisted crosses, intercrosses, and backcrosses. Our breeding approach allowed us to test whether introgression of the F344 genome proximal to *Gimap5* resulted in *1*) no effect on diabetes development or *2*) protection from diabetes. The first outcome would underscore lymphopenia and the *Gimap5* mutation as a diabetogenic factor. The alternative outcome would identify a diabetogenic factor independent of lymphopenia. The molecular identification of this genetic factor(s) should prove critical to disclose the mechanisms by which diabetes develops in the BB rat.

RESEARCH DESIGN AND METHODS

***DR.lyp* rats.** The parental DR.*lyp* (BBDR.BBDP.$^{lyp/lyp}$) line used in the present study was derived from animals with two independent recombination events developed from our previously described introgression of lymphopenia by cyclic cross-intercross breeding of BBDP with BBDR (7). The first recombination event was flanked by simple sequence–length polymorphism (SSLP) marker D4Rhw10 and the second flanked by the SSLP marker D4Rhw11. These DR rats thus have a 2-Mb fragment of DP on chromosome 4. The remainder of the genome represents BBDR as verified by genome-wide scanning (data not shown). The chromosome 4 map is shown in Fig. 1 for the DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats used in the present study.

BBDR rats. BBDR rats used to secure introgression of the recombination have been kept in sister/brother breeding for 54 generations as of December 2005.

Breeding. The one rat (Fig. 1, *lane 5*) containing the recombination event within the *lyp* critical interval that left *Gimap5* and *Gimap1* as DP and the remaining *Gimap* genes as F344 was crossed to a female BBDR. The recombination was introgressed onto the congenic DR.*lyp* line through backcrosses to DR.$^{lyp/lyp}$ (BBDR.BBDP.$^{lyp/lyp}$) and intercrosses within the line itself to check the lymphopenia and diabetes phenotypes (Fig. 2). The 33-Mb F344 genomic DNA fragment was fixed onto the DR.*lyp* background in a total of nine backcross and seven intercross matings.

Housing. Rats were housed in the K-wing specific pathogen–free facility at the University of Washington, Seattle, Washington, on a 12-h light/dark cycle with 24-h access to food (Harlan Teklad, Madison, WI) and water. The University of Washington Rodent Health Monitoring Program was used to track the infectious agent status of the BB rats. This was accomplished via a quarterly sentinel monitoring system to exclude the infectious agents listed at http://depts.washington.edu/compmed/rodenthealth/index.html#excluded. All protocols were approved by the institutional animal use and care committee of the University of Washington.

Blood lymphocyte phenotyping. Between 25 and 30 days of age, two drops of tail vein blood were obtained and diluted in 5 ml Gey's solution as described in detail by Hawkins et al. (24). The fraction of fluorescent T-cell receptor (TCR)-positive T-cells among mononuclear cells was determined on an EPICS Elite Flow Cytometer (Beckman Coulter, Fullerton, CA). Lymphopenia is expressed as mean percentage of TCR-positive T-cells ± SE.

Diabetes diagnosis. Starting at 40 days of age, all rats were weighed daily (Sartorious, Edgewood, NY) and blood glucose (Ascensia Elite XL; Bayer, Leverkusen, Germany) tested if the rat did not gain weight compared with the previous day. Diabetes was diagnosed when blood glucose exceeded 200 mg/dl for 2 consecutive days. Age at onset is shown as the mean age in days ± SE.

Histology. Pancreas and thyroid were removed and fixed in 4% (wt/vol) paraformaldehyde in PBS and stored overnight at 4°C. Samples were rinsed in 1× PBS for 1–2 h and stored in 70% ethanol before being embedded in paraffin. Sections (5 μm) were cut and stained with hematoxylin and eosin. Scoring of insulitis as well as of thyroiditis carried out on coded sections by two independent investigators was as follows (duplicate for each animal): +0, no infiltration; +1, infiltration with mononuclear cells around blood vessels or ducts; +2, occasional mononuclear cells infiltration; +3, distinct mononuclear cell infiltration; and +4, mononuclear cell infiltration with little recognizable normal tissue (23).

Genotyping

***Chromosome 4*.** Between 25 and 30 days of age, 5-mm tail snips were obtained and DNA isolated using a phenolchloroform extraction protocol as described in detail elsewhere (24). The samples were diluted to 25 ng/μl in a 10 mmol/l Tris, 0.5 mmol/l EDTA (TE) solution and 2 μl of this genomic DNA solution was used in each of the following 10-μl reactions.

Lyp region primers included the following: 1 μl of 10× reaction buffer (Promega, Madison, WI), 0.8 μl MgCl (Promega), 0.2 μl 10 mmol/l dNTPs (New England BioLabs, Beverly, MA), 0.5 μl of 1 μmol/l IRDye 700 labeled primer (LiCor Biosciences, Lincoln, NE), 0.5 μl of 20 μmol/l unlabeled reverse primer (Qiagen, Valencia, CA), 0.1 μl *Taq*DNA Polymerase (Promega), 0.04 μl of 10 mg/ml BSA (New England BioLabs, Beverly, MA), and 4.4 μl ddH_2O.

Sequence-tagged site (STS) primers outside of the lyp region included the following: 1 μl 10× reaction buffer (Promega), 0.8 μl MgCl (Promega), 0.2 μl of 10 mmol/l dNTPs (New England BioLabs), 0.5 μl of 1 μmol/l M13-labeled

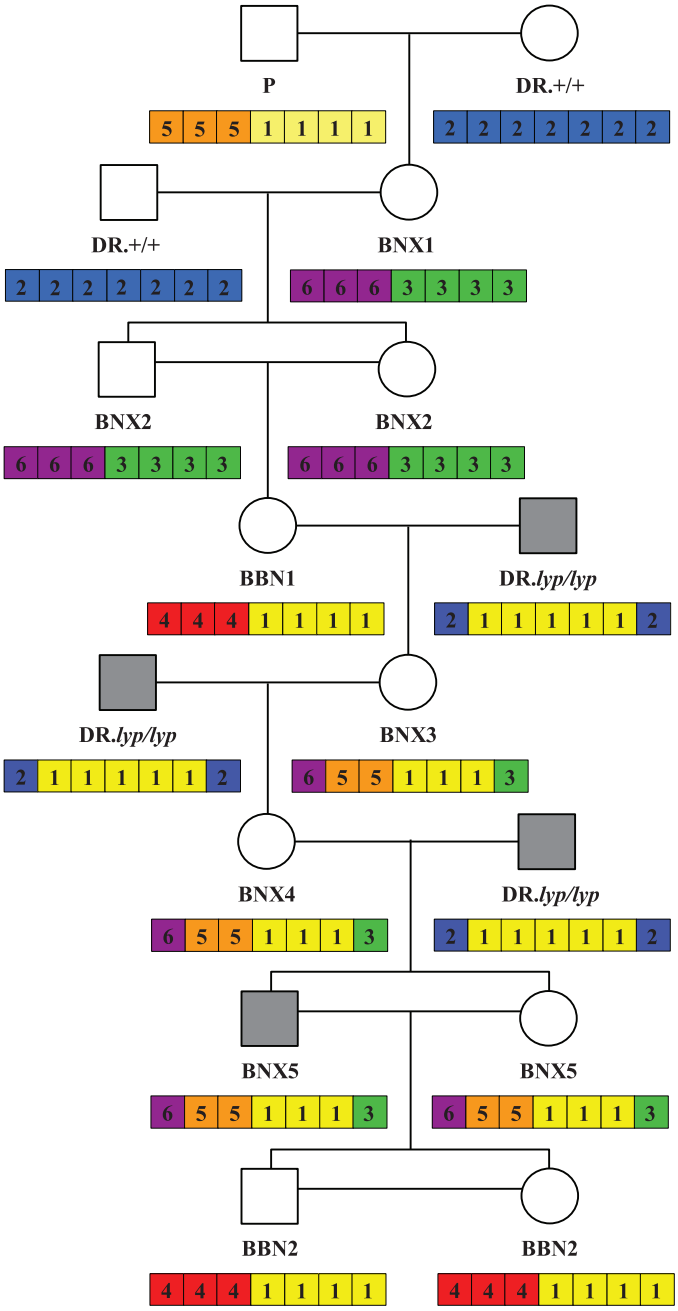

FIG. 2. *Lyp* region pedigree. Shaded boxes indicate diabetes onset. Boxes below each rat ID represents six markers used for standard colony genotyping in the *lyp* critical interval. *Left to right*: D4Rhw11, D4Rhw12, D4Rhw6, D4Rhw8, D4Rhw13, and D4Rhw10. The numerical color code is identical to that of Fig. 1; yellow (1) DP/DP, blue (2) DR/DR, green (3) DP/DR, red (4) F344/F344, orange (5) DP/F344, and purple (6) DR/F344. P is the original recombinant rat with the heterozygous DP/F344 recombination shown at D4Rhw6 and DP DNA from D4Rhw8 to the end of the chromosome (see Fig. 1 for details). BNX generations denote heterozygous recombinants produced from backcrosses with either BBDR (DR/F344 at D4Rhw6 and DP/DR from D4Rhw8 to the end of the chromosome) or DR.*lyp/lyp* (DR/F344 at D4Rhw11, DP/F344 from D4Rhw12 to D4Rhw6, DP/DP from D4Rhw8 to D4Rhw13, and DP/DR at D4Rhw10). BBN generations denote homozygous recombinants produced from intercrosses between two heterozygotes within the line and contain F344/F344 at D4Rhw6 and DP/DP DNA from D4Rhw8 to the end of the chromosome.

forward primer (Qiagen), 0.5 μl of 20 μmol/l unlabeled reverse primer (Qiagen), 0.1 μl *Taq*DNA Polymerase (Promega), 1 μl M13-700 (LiCor Biosciences), and 3.9 μl ddH_2O.

All samples were then amplified using the following standard PCR protocol: 95°C for 5 min, 95°C for 20 s, 60°C for 20 s, 72°C for 30 s, steps 2–4 repeated 30 times, then 72°C for 3 min. PCR products were diluted to 25% with STOP solution (LiCor Biosciences) and analyzed using a NEN Global IR^2 DNA Analyzer System (Model 4200S-2) with a 6.5% gel matrix (LiCor Biosciences).

Whole genome scan. To ensure that the genomic background of the new strain was fixed for BBDR, we genotyped SSLPs spanning the entire rat genome. At the N6-N8 generations (Fig. 2), we genotyped 144 SSLPs with an average genome coverage of 10 cM. At the N11 generation, the number of SSLPs was reduced to 46, including only markers on chromosomes that were not fully fixed along the entire chromosome length (at a 10 cM resolution). All SSLPs were amplified and genotypes determined using fluorescent genotyping on an ABI 377, as outlined in detail elsewhere (25). Briefly, each forward primer for the SSLP is synthesized with a 5′ tail containing the universal M13 primer sequence (5′ TGTAAAACGACGGCCAGT-SSLP-f 3′). The PCR is a two-step reaction containing the primers specific for the SSLP and an additional fluorophore-labeled M13 primer. The initial amplification steps incorporate and amplify the SSLP specific primers, thus incorporating the M13 tail. The latter rounds incorporate the labeled M13 dye-conjugate primer, allowing for detection on the ABI 377. Multiple fluorophores are used, in combination with different PCR product sizes, to allow multiplexing of six SSLPs at the gel electrophoresis level.

Each 6-μl PCR contains 30 ng genomic DNA, 200 μmol/l dNTPs, 230 nmol/l each forward and reverse primer, 150 nmol/l M13-dye conjugate primer, and 0.1 unit/μl *Taq*DNA Polymerase. Products were amplified using a touchdown PCR protocol. PCR product from six markers is then pooled and electrophoresed on a single lane of an ABI 377 and genotypes determined using the Genescan and Genotyper software (ABI).

Sequencing. Sequencing was performed with ABI BigDye Terminator v3.1 Cycle Sequencing Mix and analyzed on an ABI 3730XL sequencer at the University of Washington Biochemistry Sequencing Core.

Bioinformatics

The following resources were used: the *1*) University of California Santa Cruz Rat Genome Browser, available at http://genome.ucsc.edu/cgi-bin/hgGateway?org=rat; the *2*) Rat Genome Database, available at http://rgd.mcw.edu/; the *3*) National Center for Biotechnology Information, available at http://www.ncbi.nlm.nih.gov/; and *4*) Primer3 (Massachusetts Institute of Technology), available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

Statistical analysis. Mean values ± SE are shown as indicated. Student's *t* test was used to test for differences between groups, and $P < 0.05$ was considered significant.

RESULTS

Characteristics of the parental DR.^{lyp} rats. The DR.*lyp* congenic line developed in our laboratory contains 2 Mbp of the *lyp* critical interval introgressed onto the BBDR genome (Fig. 1). DR.$^{+/+}$ and DR.$^{+/lyp}$ rats are diabetes resistant and have normal levels of R73 TCR-positive T-cells (Fig. 3*A*). The DR.$^{lyp/lyp}$ rats have markedly reduced TCR-positive T-cells (Fig. 3*A* and *B*) and developed diabetes between 46 and 81 days of age (mean age at onset 61 ± 1 days) (Fig. 4 and Table 1). Diabetes frequency in the parental DR.$^{lyp/lyp}$ rats was 100% (Fig. 5 and Table 1).

Characteristics of the F344 introgression in DR.lyp rats. In an intercross of F1(BBDP x F344) offspring, we discovered a recombination event proximal of *Gimap1* (Fig. 1, *lane 5*). The strategy to fix the F344 genome fragment was to combine complete genotyping of the *lyp* critical region with phenotyping for lymphopenia and diabetes. Whole-genome scans, STS, and SSLP analyses (Fig. 1) showed that the F344 genome had been introgressed on rat chromosome 4 between D4Rat253 at 44.69 Mb and D4Rhw6 at 76.83 Mb. Sequencing across the right-most breakpoint revealed the recombination event to be within D4Rhw6, just proximal to *Gimap1*. The congenic line, BBDR with BBDP and the homozygous 33-Mb F344 introgression on chromosome 4, referred to as DRF.$^{f/f}$ rats have retained DP DNA (including *Gimap1* and *Gimap5*) from D4Rhw8 to the end of chromosome 4 as illustrated by marker D4Rat62 (Fig. 1, *lane 8*). During introgression of the F344 DNA fragment that generated the DRF congenic line, rats were produced that did not

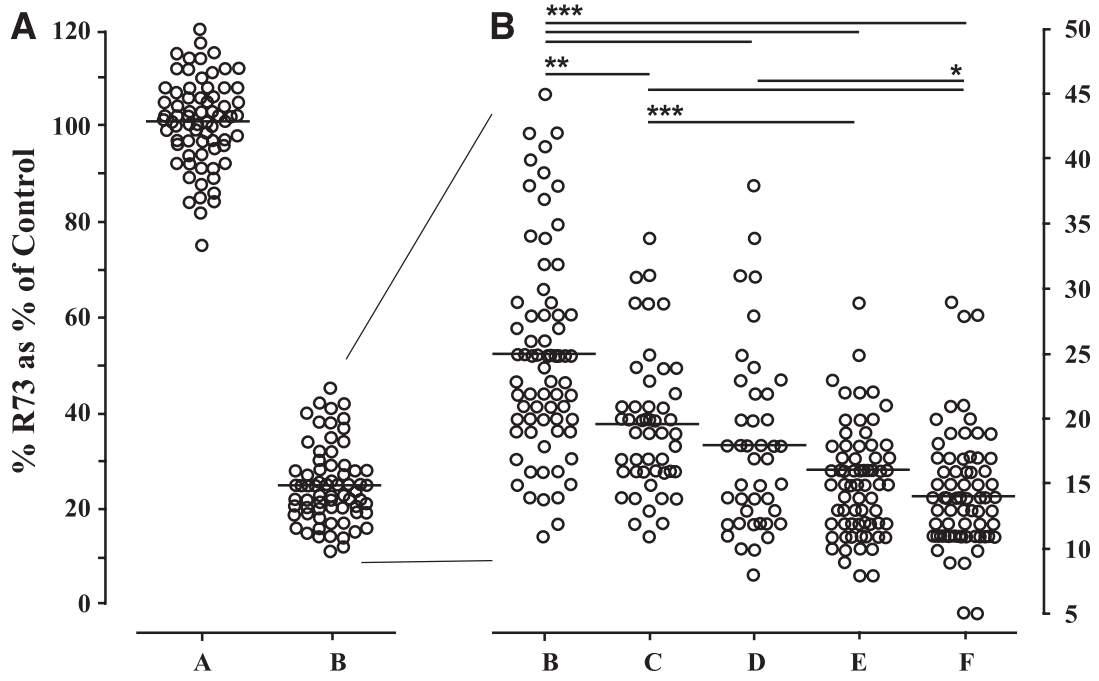

FIG. 3. R73 TCR phenotyping. Percentage of TCR-positive cells as a percentage of control. All rats were phenotyped between 25 and 30 days of age. DR.+/+ (n = 70) (A), DR.*lyp/lyp* (n = 97) (B), DRF.*b/b* (n = 40) (C), diabetic DRF.*b/f* (n = 42) (D), nondiabetic DRF.*b/f* (n = 28) (E), and DRF.*f/f* (n = 104) (F). The nondiabetic DRF.*b/f* and DRF.*f/f* were followed to between 56 and 220 days of age. Line through each group is the mean percentage of the TCR-positive level. Significance is ***$P < 0.0001$, **$P < 0.001$, and *$P < 0.05$ using Student's t test.

contain the recombination event (DRF.$^{b/b}$) (Fig. 1, *lane 6*). These rats genetically mimic the DR.$^{lyp/lyp}$ (Fig. 1, *lane 4*) and thus provided an internal control for comparison of the lymphopenia and diabetes phenotypes between the two inbred lines.

Characterization of lymphopenia. Introgression of the F344 fragment, defined by the STS markers D4Rat253 and D4Rhw6 (Fig. 1), was proximal to the *Gimap5* lymphopenia gene. The data in Fig. 3 show the percentage of TCR-positive cells detected with the monoclonal antibody R73 in the paternal DR.$^{lyp/lyp}$ and DR along with the DRF rats. The nonlymphopenic DR.$^{+/+}$ rats showed a mean of 98 ± 1 (n = 75), whereas the DR.$^{lyp/lyp}$ rats had 26 ± 1 (n = 97). Surprisingly, the DRF.$^{f/f}$ rats showed significantly lower frequency of TCR-positive cells (14 ± 1, n = 103) compared with the DR.$^{lyp/lyp}$ rats ($P < 0.0001$). Analysis of the CD3+ T-cells from spleen and mesenteric lymph node showed the same lower frequently of TCR-positive cells for DRF.$^{f/f}$ compared with DR.$^{lyp/lyp}$ rats (data not shown).

Characterization of diabetes. The mean age of diabetes onset in parental DR.$^{lyp/lyp}$ rats was 61 ± 1 days (range 46–81 days of age, n = 101 rats) (Fig. 4 and Table 1). The cumulative frequency indicates that all parental DR.$^{lyp/lyp}$ rats generated during the course of this study developed diabetes (Fig. 5). The age at onset distribution of diabetes in DRF.$^{b/b}$ rats did significantly differ ($P < 0.0001$) from the DR.$^{lyp/lyp}$ rats in that the mean age at onset was 68 ± 1 days (range 50–143 days, n = 93) (Fig. 4 and Table 1). The

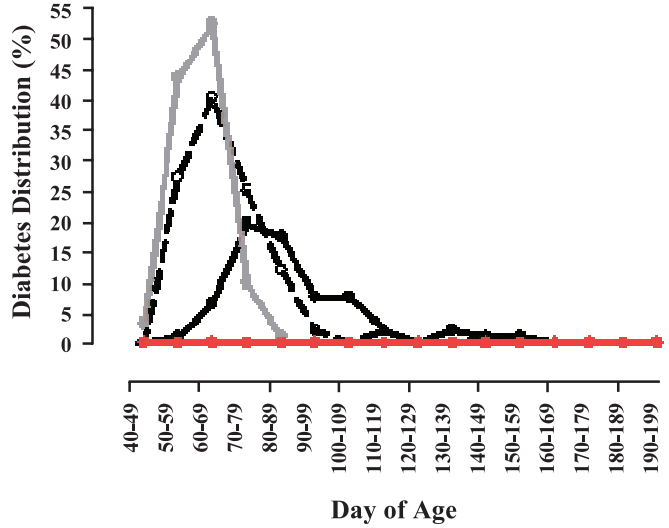

FIG. 4. Diabetes distribution. The DR.*lyp/lyp* (n = 101) rats are represented by a solid gray line, DRF.*b/b* (n = 93) by a dashed black line, DRF.*b/f* (n = 102) by a solid black line, and DRF.*f/f* (n = 85) by a solid red line.

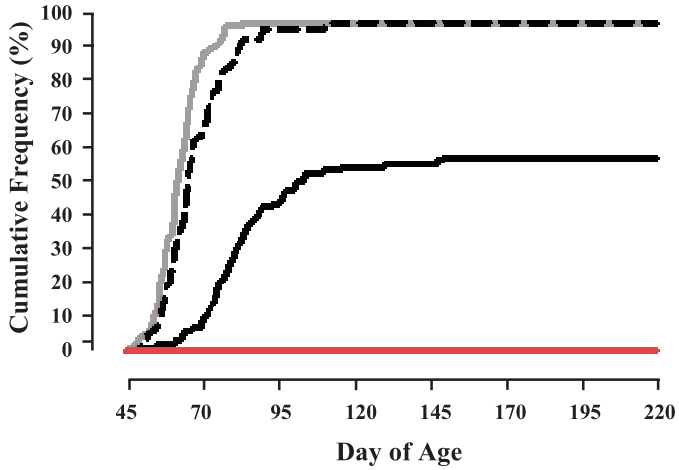

FIG. 5. Cumulative frequency. The DR.*lyp/lyp* (n = 101) rats are represented by a solid gray line, DRF.*b/b* (n = 93) by a dashed black line, DRF.*b/f* (n = 102) by a solid black line, and DRF.*f/f* (n = 85) by a solid red line.

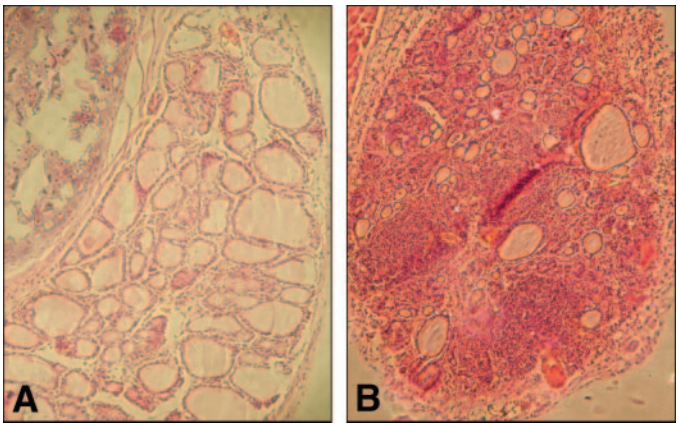

FIG. 6. Histology of the thyroid in the DRF.f/f rat. *A*: The thyroid histology was normal (score 0) in the 56-day-old DRF*.f/f* rat. *B*: Severe thyroiditis (score +4) was observed in the 163-day-old DRF*.f/f* rat. All sections were stained with hematoxylin and eosin. Original magnification is ×100 in both panels.

cumulative frequency for the DRF.$^{b/b}$ rats was also 100%. DRF.$^{b/f}$ rats in contrast showed an average age at onset of 88 ± 3 days (range 52–222 days, $n = 102$) ($P < 0.0001$). The cumulative frequency indicated that the animals heterozygous for the F344 introgression showed a diabetes frequency that was reduced to 63% (102 of 163 rats) when followed until 220 days of age in the oldest rat. None of the DRF.$^{f/f}$ rats (0 of 85) developed diabetes despite being followed until 130–220 days of age before they were killed.

Characterization of insulitis and thyroiditis. At 150 days of age, the DRF.$^{f/f}$ rats were killed and their pancreas and thyroid subjected to histological analysis. Analysis showed that 59 of 64 (92%) DRF.$^{f/f}$ rats had normal-appearing islets. Insulitis (scored +1 to +4) was observed in 5 of 64 of these rats (8%) (Table 2). In contrast, while the thyroid had a normal appearance (grade 0) in young DRF$^{f/f}$ rats (Fig. 6*A*), a total of 39 of 52 (75%) DRF.$^{f/f}$ showed thyroiditis, varying from +1 to +4 in thyroiditis score (Table 2). A 150-day-old DRF.$^{f/f}$ rat having grade 4 thyroiditis is shown in Fig. 6*B*.

DISCUSSION

The present study is the first to establish that the *Gimap5* mutation inducing lymphopenia can be disconnected from the diabetes phenotype in the BB rat. Therefore, the major conclusion is that the BB rat has a genetic factor for diabetes located on chromosome 4 between the microsatellite markers D4Rat253 and D4Rhw6 (44.55–76.83 Mb). The fact that only two genes in the Gimap family, *Gimap1* and *Gimap5*, remain BBDP downstream of D4Rhw6 further underscores the finding that the cause of lymphopenia is due to the frameshift mutation in the *Gimap5* gene (12,13). The consequence of this mutation is not only a marked reduction in *Gimap5* transcripts (13,15,26) but more importantly in an apparent complete obliteration of *Gimap5* protein expression (27). The absence of a normal gene product affirms the notion that the *Gimap5* mutation effectively is a null allele and represents a spontaneous knock-out of the *Gimap5* protein. There is no indication from genomic and cDNA sequencing of *Gimap1* that this gene contributes to the lymphopenia phenotype (E. Rutledge, B. Van Yserloo, D.H.M., R. Ettinger, J.M.F., P. Gaur, J. Hoehna, M. Peterson, R. Jensen, A.E.K., A.L., unpublished observations). It is therefore of interest that the 33 Mb of F344 genomic DNA that we introgressed onto the DR.*lyp* is, if anything, more lymphopenic than in the parental DR.$^{lyp/lyp}$ rat. However, the DRF.$^{f/f}$ congenic is completely resistant to developing diabetes, indicating that diabetes and lymphopenia are genetically distinct in the DR.*lyp* rat.

Although the BBDR rat has a number of genetic factors that make it prone to develop diabetes, there is no spontaneous disease; rather, the rats must be induced to be lymphopenic in order to develop diabetes. One example of induction is to treat the BBDR rats with immunological perturbants such as monoclonal antibodies to RT6.1, either alone (28) or in combination with polyI:C (29). Lymphopenia and subsequent diabetes can also be induced by infection with the T-cell cytotoxic Kilham rat virus (30,31). Our DR.*lyp* congenic has the *lyp* allele from the BBDP strain—a single gene defect resulting in spontaneous lymphopenia—on the nonlymphopenic BBDR genomic background (Fig. 1, DR.$^{lyp/lyp}$ in *lane 4*). Therefore, congenic DR.$^{lyp/lyp}$ have the same diabetes susceptibility factors found in the BBDR, with spontaneous lymphopenia due to the genetic defect in *Gimap5*. These observations have lead to the notion that, while spontaneous diabetes is controlled by the *Gimap5* null allele, the BBDR rat has all genetic factors necessary for diabetes development; it simply requires immunological perturbants to develop disease (32). In contrast, our DR.$^{lyp/lyp}$ rats are 100% lymphopenic and all develop diabetes between 46 and 81 days of age in our current generations. Hence, all the genetic factors necessary for spontaneous diabetes development are present in our congenic DR.*lyp* rat line. In the double-congenic DRF.$^{f/f}$ rat, the replacement of a 33-Mb region upstream of *Gimap5* renders the animal severely lymphopenic but resistant to spontaneous diabetes. This demonstrates that a gene(s) in this interval is required for diabetes development, independent of lymphopenia.

Recent studies by Martin et al. suggest that the *Iddm4* locus is bounded by the proximal marker D4Rat135 and the distal marker D4Got51 (61.51–67.35 Mb). It is interesting to note that the *Iddm4* locus is contained in the introgressed region of the DRF rat (18). It is therefore tempting to speculate that the genetic factors required for diabetes in the DRF line are allelic to *Iddm4*. The approach to map *Iddm4* differs from our approach to dissect the spontaneous onset type of diabetes, but the present study may be of help to identify hypothetical genetic factors in *iddm4* independent of modifying genetic factors. Indeed, after inducing diabetes with the Kilham rat virus, a parvovirus cytopathic to T-cells but not β-cells, it was reported that not only did diabetes segregate with *Iddm4* but also with a locus on chromosome 17 (*Iddm20*) (33). Similar experiments in the lymphopenic and diabetes-resistant DRF.$^{f/f}$ congenic line would complement these studies, as these animals are already severely lymphopenic due to the null *Gimap5* allele. The previous studies of the *Iddm4* locus were done in the BBDR rat in crosses with Wistar-Furth (WF) rats. It is likely that WF rats also carry a resistance allele, and it will be of interest to compare WF and F344 candidate gene sequences in the further dissection of the diabetes resistance factor discovered in the present investigation.

The mechanisms by which the *Gimap5* null allele causes lymphopenia remains to be determined. The identification of the *Gimap* family of proteins and the potential to generate specific antibody reagents (27) should make it feasible to better dissect the series of events that preclude

the survival of T-cells past the thymus. It cannot be excluded that cellular events preceding the spontaneous onset of diabetes may compromise the function of T-cells that survive the *Gimap5* null allele (34). If that is the case, however, the process would have to be specific for diabetes, since all of the DRF.$^{f/f}$ rats that we followed until 150 days developed thyroiditis, while insulitis was conspicuously absent. Two recent studies have shown that adoptive transfer of DR.$^{+/+}$ CD4+CD25+ T-cells to DR.$^{lyp/lyp}$ rats protect the recipients from diabetes (35,36). It is possible that such cells regulating diabetogenesis may be affected by the *Gimap5* null allele more than other thymic T-cells (35). The congenic DRF.$^{f/f}$ rats should be particularly instrumental in this regard, since these animals are severely lymphopenic but do not develop diabetes, therefore proving useful in studies of these and other T-cell subsets in the absence of signals or events eventually leading to diabetes.

Although fewer studies have been published on the lymphocytic thyroiditis in the BB rat, compared with insulitis and diabetes, several important observations have been made. First, spontaneous lymphocytic thyroiditis varies between different DP sublines (37). Second, interleukin-1β treatment induced thyroiditis in both DP and DR rats (38). Based on crosses between BBDR, BBDP, Lewis, and F344 rats, we showed that two susceptibility factors for diabetes, the *Gimap5* null allele and the MHC, also appeared to be risk factors for thyroiditis (39). While the *Gimap5* null allele was absolutely required for diabetes, it only conferred risk for thyroiditis. Also, in contrast to diabetes, the MHC RT1.B$^{u/u}$ conferred dominant susceptibility to thyroiditis (9,39). The DRF.$^{f/f}$ congenic rats support this notion, since they developed thyroiditis in the presence of lymphopenia but in the absence of diabetes. It is of interest in this regard that the 33-Mb F344 genomic DNA that has been introgressed in the DRF.$^{f/f}$ congenic line would harbor the *Iddm4* locus previously mapped to rat chromosome 4 following a cross between BBDR and WF rats (32) and induction of diabetes after treatment with polyinosinic:polycytidylic acid and an antibody that depletes ART2(+) regulatory cells (18). These data suggest that it should be possible in the future to map both MHC and non-MHC genetic factors in the DRF.$^{f/f}$ rats that are important to the spontaneous development of thyroiditis.

We conclude from the present rescue of a recombination event within the critical lymphopenia region in a cross between BB DR.*lyp* and F344 rats that *1*) the *Gimap5* null allele is sufficient to induce severe lymphopenia, *2*) introgression of 33-Mb F344 genomic DNA proximal to the *Gimap1* and *Gimap5* genes confers diabetes resistance, *3*) BBDR and BBDP rats carry susceptibility and F344 rats carry resistance factor(s) for diabetes, *4*) further cross-intercross-backcross analyses of DR and F344 recombinants should make it possible to positionally clone the diabetes genetic factors, and *5*) it is now possible to test the hypothesis that the *Iddm4* and the present diabetes susceptibility locus are allelic. The present data underscore the importance of the BB rat in dissecting the genetics of spontaneous autoimmune diabetes and should prove useful in developing novel approaches to diagnoses and therapy for human type 1 diabetes. We predict that the diabetes susceptibility locus upstream of the *Gimap5* null allele will provide a better understanding of the mechanisms by which the MHC in particular is controlling the specific age-dependent spontaneous development of insulitis as well as thyroiditis.

ACKNOWLEDGMENTS

This study was supported by the National Institutes of Health (grant AI042380), the Robert H. Williams Endowment, and the Juvenile Diabetes Research Foundation. Daniel Moralejo was supported by an American Diabetes Association Junior Faculty Award.

The assistance of Sara Speros, Will Simonson, Ruth Ettinger, Phil Yen, Matt Hillman, Mark Tenforde, Sahar Manavi, and Ranae Pefley with the BB rat colony throughout the 6 years it took to develop the DRF.$^{f/f}$ congenic line is much appreciated.

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