Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4062–4066, April 1994
Immunology

Genetic analysis of the NZB contribution to lupus-like autoimmune disease in (NZB × NZW)F_1 mice

(systemic lupus erythematosus/linkage/New Zealand mice)

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Communicated by Philippa Marrack, January 18, 1994

ABSTRACT **Lupus-like autoimmunity in (NZB × NZW)F_1 mice is frequently marked by the development of a severe and fatal renal disease. Genes from both NZB and NZW parents are required for the full expression of disease. We applied a mapping technique based on polymorphism in simple sequence repeats to the analysis of (NZB × NZW)F_1 × NZW backcross mice to determine the NZB genetic contribution to disease. The results show that a single NZB locus or tightly linked group of loci on the distal part of chromosome 4 provides the strongest association with renal disease and death. This locus, designated here as *nba-1* (New Zealand Black autoimmunity), lies distal to the locus *elp-1*, 60–70 centimorgans from the centromere. It is of interest that a gene encoding a receptor for tumor necrosis factor maps to the vicinity of this disease-associated gene.**

(NZB × NZW)F_1 mice spontaneously develop an autoimmune process remarkably similar to that seen in the human disease systemic lupus erythematosus. F_1 females produce IgG antinuclear autoantibodies, including IgG antibodies to double-stranded DNA, and develop a severe immune-complex-mediated glomerulonephritis. Greater than 95% of these mice die before 12 months of age from renal failure. Since the predominant disease characteristics of the (NZB × NZW)F_1 mouse are present in neither parental strain, it seems likely that genes from both NZB and NZW mice act in concert for full expression of the autoimmune phenotype. Our previous work indicates that the most important NZW contribution to disease is a single dominant locus that is probably encoded within the NZW major histocompatibility complex (MHC) (1, 2). More recent congenic studies (3) directly implicate the class II MHC genes.

The NZB genetic contribution to disease, however, is less clear. Studies from several groups have suggested that the involvement of one to six unlinked NZB genes may be required for disease induction (4–7). To clarify this NZB influence, we bred (NZB × NZW)F_1 × NZW backcross mice and analyzed their genotype using a mapping technique based on simple sequence length polymorphisms (SSLPs) (8). The large number of simple sequence repeats spaced throughout the genome and the relatively high level of polymorphism between different laboratory strains make such a mapping approach feasible. This technique permits the identification of multiple loci contributing to a given trait, without a preexisting knowledge of candidate loci or positions. A similar mapping approach has been applied to the identification of disease-associated genes in nonobese diabetic mice (9, 10), a murine model of diabetes.

MATERIALS AND METHODS

Mice. Parental NZB/BINJ and NZW/lacJ mice were obtained from The Jackson Laboratory and were maintained in the animal care facility at the National Jewish Center for Immunology and Respiratory Medicine (Denver). F_1 and backcross animals were bred in this facility, and all groups of mice used in the analysis were housed in the same room and fed an identical diet. Cohorts of parental NZB and NZW mice were followed concomitantly and also evaluated for expression of renal disease and death. Only female animals were utilized in the present experiments.

Evaluation of Renal Disease. Mice were evaluated monthly for proteinuria using tetrabromphenol paper (Chemstrip; Boehringer Mannheim). Urine samples were graded colorimetrically from 1^+ to 4^+, corresponding to approximate protein concentrations as follows: 1^+, ≈30 mg %; 2^+, ≈100 mg %; 3^+, ≈300 mg %; 4^+, ≥1000 mg %. Mice with negative or trace determinations of proteinuria and no clinical evidence of disease at 12 months of age were designated as not expressing lupus-like renal disease (negative phenotype). Mice with severe (2^+ or greater) proteinuria on two or more separate occasions before 12 months of age were designated as positive for disease. Nearly all of the diseased mice died before 12 months of age, usually within 2 months of the development of severe proteinuria. A few severely ill and wasted animals with evidence of renal disease were sacrificed prior to death for collection of tissues.

Tissue and DNA Samples. Liver and kidney samples were collected from positive mice at the time of sacrifice or death and from negative mice upon sacrifice at 12 months of age. Samples were frozen at −70°C, after which DNA was extracted as described (11). Based upon the availability of genomic DNA, 30 mice from each group were selected for genetic analysis.

PCR Amplification. Oligonucleotides flanking simple sequence repeats were either purchased (Research Genetics, Huntsville, AL) or synthesized at the National Jewish Molecular Resource Center by using an Applied Biosystems model 392 DNA synthesizer. The sequences for these oligonucleotide primers have been described (8, 9, 12–16). PCR amplification was performed in a PTC-100 model 96 (96 well) thermal cycler (MJ Research, Watertown, MA). PCRs generally utilized 35 cycles of 1 min at 94°C, 1 min at 55°C, and 30 sec at 72°C. Reactions were performed in a total volume of 25 μl—all remaining reaction conditions were as described (8).

Analysis of PCR Products. After amplification, 10–15 μl of PCR product was loaded onto a 1.0-mm-thick 10-cm-long 15% polyacrylamide gel (Bio-Rad MiniProtean II) and electrophoresed between 2 and 4.5 h at a constant voltage of 100–120 V. After ethidium bromide staining, individual back-

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Abbreviations: MHC, major histocompatibility complex; SSLP, simple sequence length polymorphism; cM, centimorgan(s); TNF-RII, tumor necrosis factor receptor.

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cross animals were scored as BW (heterozygous) or WW (homozygous) for a particular marker by comparison with simultaneously amplified genomic samples from NZB(BB), NZW(WW), and F_1 (BW) strains.

Statistics. The association of a particular locus with renal disease was quantified by χ^2 analysis, using a standard 2 × 2 contingency matrix (17). Values >3.8, corresponding to P values of <0.05, were considered significant. No correction for multiple comparisons was applied so that all loci showing statistically significant associations with disease expression would be highlighted.

RESULTS

Expression of Renal Disease in (NZB × NZW)F_1 × NZW Backcross Mice. To determine the NZB genetic contribution to disease in (NZB × NZW)F_1 autoimmune mice, appropriate backcross animals [(NZB × NZW)F_1 × NZW] were generated and screened for proteinuria as described above. Because of the well-described gender influence on disease development (18–20), only female mice were included in this evaluation. Concomitant cohorts of parental strain mice, (NZB × NZW)F_1 mice, and backcross mice were followed for 1 year. Table 1 shows that 6.2% of NZB females and none of the NZW females displayed evidence of severe renal disease. In contrast, 86% of the (NZB × NZW)F_1 mice developed severe renal disease and died prior to 1 year of age during the study period. These results are consistent with previous studies from our laboratory (1) and others (21). Approximately 48% of the (NZB × NZW)F_1 × NZW backcross mice showed evidence of severe renal disease. These data are consistent with the hypothesis that a single dominant NZB locus is of primary importance in the expression of lupus-like renal disease. However, the data may also reflect a process in which multiple threshold genetic influences combine to produce a polygenic trait.

Genetic Analysis of (NZB × NZW)F_1 × NZW Backcross Mice. To localize the NZB gene(s) involved in autoimmune renal disease, 60 of the backcross animals were selected for further genetic analysis. Thirty animals with clear evidence of disease (≥3$^+$ proteinuria before 1 year of age) were included in the "positive" group, and 30 animals that had survived for 1 year with no evidence of renal disease were randomly chosen for the "negative" group. The parental NZB and NZW strains are laboratory inbred, and hence, homozygous at every locus. These genotypes are designated as BB and WW, respectively. The F_1 animals are therefore heterozygous (BW), and the F_1 × NZW backcross animals are either BW or WW at any given genetic locus. If a particular genetic marker is in the proximity of a gene involved in the disease process, one expects a preponderance of heterozygous (BW) animals in the disease group.

Backcross mice were genotyped using a method based on PCR amplification of simple sequence repeats, or microsatellites, originally described by Love *et al.* (8). Approximately 350 markers were screened for polymorphism between the NZB and NZW strains to obtain the set of informative markers used in the analysis. An example of the way in which a SSLP was used to genotype backcross mice is shown in Fig. 1. Table 2 shows a summary of the results obtained with 74 informative markers that span the entire genome. Using a χ^2 value >3.8 (P < 0.05) as a cutoff, three chromosomal regions showed a significant association with renal disease. The first is on the proximal segment of chromosome 2, in the vicinity of the marker *D2MIT5*. This association, though statistically significant, should be interpreted in light of the fact that a total of 74 informative loci were evaluated statistically and, by chance, some markers may show a weak statistical correlation with the disease phenotype. Similarly, there is also a weak association distal to the MHC locus on chromosome 17 (χ^2 = 4.29; P < 0.05). Of much greater significance, however, are results with markers on distal chromosome 4. For multiple loci >50 centimorgans (cM) distal to the centromere, there is a strong correlation of the BW genotype with disease expression and of the WW genotype with the absence of disease. The most significant association with disease is at the locus *elp-1* (Table 2).

Ordering of Distal Chromosome 4 Loci. To more precisely define the position of the disease-associated NZB gene on distal chromosome 4, the loci used in the genetic analysis were ordered based on their recombination frequencies (Fig. 2). The position of each locus was determined by using *lck*, at relative position 54 (22), as an anchor locus. The order of loci determined here, as well as the interlocus distances, correspond well with that determined by Dietrich *et al.* (15). Three loci (*D4Nds2*, *D4MIT16*, and *D4MIT11*) have been placed in a single relative position, since no recombination events were observed between them. Distal to these loci are *lck* and *elp-1*. The most distal marker thus far available, *D4MIT48*, shows a weaker association with disease than the *elp-1* locus, indicating that the actual disease-associated locus probably lies between *elp-1* and *D4MIT48*. We have named this putative disease-associated locus *nba-1* (New Zealand Black autoimmunity) and have tentatively placed it 7 cM distal to *lck*. This placement is based on seven individual backcross mice that show evidence of a recombination event between *elp-1* and *D4MIT48*. *Nba-1* was hypothetically positioned between these loci such that there would be a

Table 1. Incidence of severe renal disease in (NZB × NZW)F_1 × NZW backcross mice

Strain	Incidence of severe renal disease/number of mice analyzed	% mice with severe renal disease
NZB	2/32	6.2
NZW	0/21	0
(NZB × NZW)F_1	31/36*	86
(NZB × NZW) × NZW	42/88†	48

Only female animals were followed for expression of disease in this study.

*Two additional animals were noted to have severe proteinuria at 12 months but were not followed further.

†Nine additional animals could not be phenotyped as either positive or negative for disease and were excluded from further analysis.

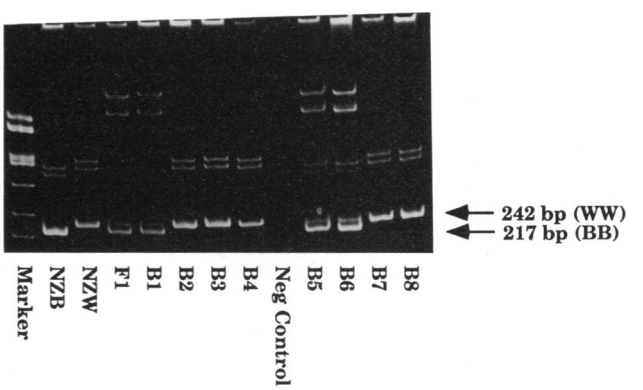

FIG. 1. Genotyping of backcross mice based on SSLP. Genomic DNA from NZB, NZW, (NZB × NZW)F_1, and individual backcross animals (designated B1–B8) was amplified using PCR primers specific for the simple sequence repeat *D3MIT22*, as described (8). PCR products were separated on a 15% polyacrylamide gel and stained with ethidium bromide. Amplification of NZB genomic DNA gives a product of ≈217 bp, and amplification of NZW DNA yields a product of ≈242 bp. Backcross animals were typed as either homozygous for the NZW allele (WW) at this locus or heterozygous (BW) by comparison with the banding patterns in the control lanes. Thus, mice B1, B5, and B6 are heterozygous (BW) at this marker, and mice B2, B3, B4, B7, and B8 are homozygous for the NZW allele (WW).

Table 2. Analysis of genotypes in (NZB × NZW)F_1 × NZW backcross mice

Locus	Position	Positive mice, no. WW	Positive mice, no. BW	Negative mice, no. WW	Negative mice, no. BW	χ^2	*P* value
D1Nds4	1,17	17	13	16	14	0.07	
D1Nds1	1,52	11	10	11	13	0.19	
Crp	1,72	9	8	7	10	0.47	
D2MIT5	2,6	9	21	18	12	5.45	<0.05
D2MIT8	2,30	10	19	16	14	2.13	
D2MIT10	2,42	13	16	12	17	0.07	
D2Nds1	2,45	15	15	11	18	0.87	
D2MIT28	2,68	16	10	11	16	2.29	
D3MIT1	3,35	13	17	12	18	0.07	
D3MIT22	3,52	12	18	14	16	0.27	
D3Nds2	3,77	12	18	13	16	0.14	
D3MIT19	3,100	12	18	12	18	0.00	
D4MIT1	4,16	18	12	15	15	0.61	
D4MIT17	4,31	11	19	14	16	0.62	
Orm-1	4,31	10	20	14	16	1.11	
D4MIT9	4,41	10	16	16	12	1.88	
D4MIT15	4,41	12	18	17	13	1.67	
D4Nds2	4,53	9	21	20	10	8.08	<0.005
D4MIT11	4,53	9	21	20	10	8.08	<0.005
lck	4,54	8	22	21	9	11.28	<0.001
D4MIT16	4,55	9	21	20	10	8.08	<0.005
elp-1	4,58	8	22	22	8	13.07	<0.0005
D4MIT48	4,67	9	21	22	8	11.28	<0.001
Il-6	5,11	16	14	17	13	0.07	
D5MIT6	5,40	11	14	10	13	0.00	
Zp-3	5,71	15	15	17	13	0.27	
D6MIT8	6,35	16	13	13	17	0.83	
D6MIT10	6,53	15	12	15	15	0.18	
D6MIT13	6,60	15	15	13	17	0.27	
D7Nds5	7,22	11	12	12	12	0.02	
D7MIT16	7,39	15	13	15	12	0.02	
D7MIT7	7,51	14	14	15	11	0.32	
D7Nds4	7,75	16	14	17	13	0.07	
D8MIT16	8,5	7	22	11	18	1.29	
D8MIT6	8,27	9	15	12	14	0.38	
D8MIT13	8,69	10	20	17	12	3.80	
D9MIT23	9,24	18	12	18	12	0.00	
D9MIT11	9,42	17	13	16	14	0.07	
D9MIT17	9,68	11	19	16	14	1.68	
D10MIT15	10,23	15	11	12	12	0.30	
D10MIT8	10,50	14	14	15	15	0.00	
D10MIT11	10,58	18	11	15	14	0.63	
D10MIT14	10,74	18	12	16	14	0.27	
D11MIT16	11,3	18	12	11	19	3.27	
D11Nds9	11,30	15	15	9	21	2.50	
D11Nds4	11,44	12	18	12	18	0.00	
D11Nds7	11,59	13	16	12	18	0.14	
Empb-3	11,68	14	15	12	18	0.41	
D12MIT2	12,18	11	13	12	12	0.08	
D12MIT7	12,50	11	18	16	11	2.55	
D13MIT1	13,17	15	13	8	16	2.15	
D13MIT9	13,49	15	15	13	15	0.07	
D14MIT1	14,1	18	12	15	15	0.61	
D14MIT4	14,12	16	14	15	15	0.07	
D14MIT5	14,15	16	13	13	16	0.62	
D14MIT41	14,28	18	8	14	15	2.47	
D15MIT17	15,8	15	14	14	15	0.07	
Hox-3	15,48	13	15	13	16	0.01	
D15MIT15	15,53	14	15	14	16	0.02	
D16MIT5	16,39	15	12	10	17	1.86	
D16MIT6	16,51	18	9	11	14	2.70	
D17MIT16	17,18	13	17	20	10	3.30	
TNF-α	17,19	13	17	20	10	3.30	
H-2	17,19	13	17	20	10	3.30	
D17MIT10	17,24	12	18	20	10	4.29	<0.05
D17MIT6	17,27	13	17	19	11	2.41	
D17MIT2	17,48	14	16	12	18	0.27	
D18MIT14	18,24	14	15	15	14	0.07	
D18MIT8	18,49	20	10	13	15	2.42	
D18MIT16	18,66	15	10	12	15	1.26	
D19MIT13	19,16	17	13	16	13	0.01	
D19MIT11	19,18	17	12	15	14	0.28	
D19MIT10	19,23	14	16	15	14	0.15	
D19MIT6	19,42	17	9	20	10	0.01	

Positions of the markers are designated by chromosome and centimorgans distal to the centromere. Relative positions are taken from ref. 22. In general, 30 positive mice and 30 negative mice were typed at each locus. For occasional markers, certain individual genotypes were not discernible. For such markers, data for less than the total group are presented. χ^2 values are calculated using a 2 × 2 contingency matrix as described (17). *P* values are based upon one degree of freedom in the observed data. H-2 was typed by both SSLP and restriction fragment length polymorphism techniques. The restriction fragment length polymorphism utilized *Pvu* II-digested genomic DNA and a probe for $I\text{-}A^z$ as described (2). The genotypes determined by these two techniques correlated perfectly.

maximal correlation with disease expression. Although far from perfect, the association of the NZB allele of *nba-1* with disease is striking; 23 out of 30 animals with disease carry this allele. Additionally, 24 out of 30 disease-free animals are homozygous for the NZW allele at this putative locus.

DISCUSSION

The incidence of severe renal disease in the (NZB × NZW)F_1 × NZW backcross mice in this study was very close to 50% and was approximately half of the incidence observed in (NZB × NZW)F_1 mice. In contrast, Shirai and coworkers (5, 6) found lower levels of disease expression and concluded that two nonlinked NZB genes were involved in autoimmune disease induction. Our results are also difficult to reconcile with those of Raveche *et al.* (7), who, using recombinant inbred strains, concluded that at least six unlinked NZB genes were involved in the autoimmune phenotype observed in these mice—although these studies neglected the contribution of NZW MHC loci to renal disease. The reasons for these discrepancies are not clear. However, our results are in good agreement with the work of Knight *et al.* (4, 23), who

	D4MIT1	D4MIT17	Orm-1	D4MIT9	D4MIT15	D4Nds2	D4MIT16	D4MIT11	lck	Elp-1	nba-1*	D4MIT48
D4MIT1	-											
D4MIT17	27%	-										
Orm-1	28%	2%	-									
D4MIT9	38%	12%	13%	-								
D4MIT15	37%	10%	12%	2%	-							
D4Nds2	47%	23%	22%	15%	13%	-						
D4MIT16	47%	23%	22%	15%	13%	0%	-					
D4MIT11	47%	23%	22%	15%	13%	0%	0%	-				
lck	50%	27%	25%	18%	17%	3%	3%	3%	-			
Elp-1	52%	28%	27%	20%	18%	5%	5%	5%	2%	-		
nba-1*	53%	30%	28%	25%	23%	10%	10%	10%	7%	5%	-	
D4MIT48	57%	37%	35%	32%	30%	17%	17%	17%	13%	12%	7%	-
Calculated Position	4	27	29	36	37	51	51	51	54	56	61*	67
χ^2	0.61	0.62	1.11	1.88	1.67	8.08	8.08	8.08	11.28	13.07	19.29*	11.28

FIG. 2. Ordering of chromosome 4 loci by recombination frequencies. The top portion of the figure shows recombination frequencies (in percentages) between the various markers. The bottom portion shows the position of each locus, in centimorgans, by using the locus *lck* at relative position 54 (22), as an anchor locus. The χ^2 value at each marker is also shown. Since *D4MIT48*, the most distal marker available, shows a weaker association with disease than *elp-1*, we postulated that the actual disease-associated locus, *nba-1*, lies between *elp-1* and *D4MIT48*. Based on individual backcross mice that must have a recombination event between *elp-1* and *D4MIT48*, we positioned *nba-1* between these markers such that there would be a maximal correlation with disease expression. The asterisk indicates that *nba-1*, its position, and correlation with disease (χ^2) are hypothetical based on the above prediction.

performed a similar backcross analysis and postulated that a single dominant NZB gene is required for the development of severe renal disease in (NZB × NZW)F_1 mice.

Taken alone, the observed frequency of disease in the backcross mice is insufficient evidence from which to deduce the involvement of a single NZB locus in the autoimmune process. Clearly, renal disease in these animals might stem from the involvement of a number of unlinked genes in particular combinations—a situation similar to that thought to exist in the nonobese diabetic mouse model of diabetes (9, 10). However, when we performed an extensive genetic analysis of the backcross mice, evaluating 74 markers in 60 individual animals, the results strongly supported the concept of a single dominant NZB gene (or closely linked group of genes) of primary importance in disease development. Thus, we found several markers on distal chromosome 4 for which the presence of the NZB allele corresponded well with the development of severe renal disease. Additionally, the absence of the NZB allele at these loci correlated with a failure to develop disease. Such strong correlations in both directions would not be expected if more than one NZB locus contributed in a major way to disease development. Careful ordering of these markers allowed us to tentatively position the putative disease-associated locus (*nba-1*) slightly distal to the *lck* locus. However, it should be emphasized that there is an imperfect correlation of the chromosome 4 locus with disease and that several markers in this region correlate similarly. Hence, the exact position of *nba-1* on distal chromosome 4 remains to be clarified. Interestingly, previous work by Knight and Adams (24) suggested that a single NZB gene linked to coat color and located on chromosome 4 was involved in the autoimmune hemolytic anemia typically observed in these mice.

Clearly, the chromosome 4 locus does not explain all cases of autoimmune renal disease in this model. There remains a significant number of mice who are genotypically WW at *nba-1* yet still develop renal disease. This discordance should be considered in light of the frequency of disease expression in the parental and F_1 animals. For example, only 86% of the concomitantly followed (NZB × NZW)F_1 animals developed severe disease in this study. Further, NZW and NZB animals are not entirely free from disease; an occasional animal of each strain develops severe renal disease (1). Thus, a perfect correlation with disease expression would not be expected at any NZB or NZW locus (2). This discordance may also reflect the action of yet unidentified NZB and/or NZW background genes that modify the course of disease. Indeed, the observations of Raveche *et al.* (7) with recombinant inbred strains may be due to the presence of such modifying NZB genes. The data presented here hint that other loci might lie on proximal chromosome 2 or chromosome 17, but a far greater number of animals must be analyzed before any conclusions can be drawn. Our analysis concerning linkage with disease was based on the assumption of independent assortment of contributing genes. We also examined the potential for a relationship between H-2 haplotype and the association of disease with chromosome 4 markers. Although the numbers were too small to draw definite conclusions, no such influence was apparent—i.e., the same correlation with disease held in both the $H\text{-}2^{d/z}$ and the $H\text{-}2^{z/z}$ groups. Finally, it remains possible that our genetic analysis missed important loci of interest. We estimate that our set of 74 markers covers 75–85% of the mouse genome, leaving open such a possibility.

This backcross also indirectly addressed the requirement for heterozygosity at the MHC locus, which has been implicated as an important contributor to disease expression in (NZB × NZW)F_1 mice (25). For example, previous studies using MHC congenic strains showed that (NZB × NZW)F_1 mice expressing $H\text{-}2^{d/z}$ were more likely to develop renal disease than either $H\text{-}2^{z/z}$ or $H\text{-}2^{d/d}$ animals (25). In our backcross mice, nearly one-half of the animals were heterozygous for the NZB MHC $H\text{-}2^d$ haplotype whereas the other half were homozygous for the NZW haplotype $H\text{-}2^z$. Interestingly, heterozygosity at the MHC locus was clearly not required for disease development as nearly half of the disease-positive mice analyzed were homozygous for the NZW MHC haplotype. Indeed, we could not document a significant NZB genetic contribution at this position, which was genotyped by both SSLP and restriction fragment length polymorphism techniques. The reason for this discrepancy in results is unclear. It is conceivable that an unknown environmental factor in our colony may override the requirement for a particular MHC-linked gene. It should be pointed out that the most important NZW contribution to autoimmune disease in F_1 mice lies within the MHC (1–4, 6, 25). The current work clearly shows that the NZB MHC does not contribute in a similar manner. Nevertheless, our data do support the possibility of a weak contribution from an NZB locus that is slightly distal to the MHC. Analysis of a larger set of backcross mice could help to clarify this contribution.

The mapping data presented permit us to speculate that the NZB locus involved in disease production probably lies

slightly distal to *elp-1*. Of the genes already mapped to this region (22, 26), the p75 tumor necrosis factor receptor (TNF-RII) is an attractive candidate for involvement in autoimmune disease. TNF-RII belongs to a family of receptors whose members include Fas and the receptor for nerve growth factor (27). *lpr*, which functions as an accelerator of lupus-like renal disease in the MRL-*lpr/lpr* mouse model of autoimmune renal disease, has recently been recognized as a mutant allele of *fas* (28, 29). This family of cell surface receptors, originally identified through sequence homology (27), are able to mediate apoptosis after receptor ligation (30–32). Interestingly, several groups have also reported that administration of recombinant tumor necrosis factor α to (NZB × NZW)F_1 mice alleviates the severity of autoimmune disease (33, 34). Thus, we view the potential involvement of an altered NZB TNF-RII allele in lupus-like renal disease as an important possibility. Determination of potential sequence differences and an analysis of the relative levels of TNF-RII expression may help to support this hypothesis.

We thank Sarah Burell, Trinh Cao, and Shad Eubanks of the Molecular Resource Center for the synthesis of many of the oligonucleotides used in this study, and Virginia Appel and N. Pamela Smarnworawong for their help in the preparation of genomic DNA and analysis of backcross animals. This work was supported by Grant R01-37070 from the National Institutes of Health.

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