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Genetic Approach to Study Lupus Glomerulonephritis

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Abstract

Genetic and environmental factors contribute in the pathogenesis of systemic lupus erythematosus (SLE). Lupus nephritis, the most common and severe manifestation of SLE, involves inflammation in the kidney leading to loss of renal function. However, it is not clear what controls the progression of lupus nephritis; this is an important research question, considering its implications in clinical treatment of lupus nephritis. Finding genes that underlie the development and progression of lupus nephritis will shed light on this question. NZM2328 is a spontaneous mouse model for SLE. Most NZM2328 female mice develop autoantibodies (e.g., antinuclear antibody and anti-dsDNA antibody), glomerulonephritis (GN), and severe proteinuria between 5 and 12 months of age. In contrast, C57L/J mice fail to exhibit similar signs of autoimmune disease. We used classical genetics to map and identify SLE genes in offspring generated by backcrossing C57L/J to NZM2328. Quantitative trait loci (QTL) controlling acute (*Agnz1* and *Agnz2*) and chronic (*Cgnz1*) GN features were uncovered by the analysis. To verify the *Cgnz1* and *Agnz1* on distal mouse chromosome 1, we produced the NZM2328.C57Lc1 (Lc1) congenic strain, which replaced NZM2328 *Cgnz1* and *Agnz1* alleles with those derived from C57L/J. The development of acute GN and chronic GN was markedly reduced in Lc1 mice, confirming the linkage findings. Further mapping by the generation of intrachromosomal recombinants of NZM2328.Lc1 support the thesis that acute GN and chronic GN are under separate genetic control.

Keywords

Congenic strain; End-stage renal disease; Genetic mapping; Genotyping; Glomerulonephritis; Linkage analysis; Lupus nephritis; Marker-assisted selection protocol; Microsatellite marker; NZM2328; Quantitative trait loci; QTL mapping; Speed congenic; Systemic lupus erythematosus

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease; it is characterized by autoantibody production and complement-fixing immune complex deposition that result in tissue inflammation and damage. Lupus nephritis is the most common and severe manifestation of SLE, affecting all renal compartments (i.e., glomeruli, tubules, interstitium, and renal blood vessels). Lupus nephritis contributes significantly to the morbidity and mortality of SLE patients because of the risk of progressing to end-stage renal disease (ESRD). Sixty percent of SLE patients develop lupus nephritis at some stage of the disease (1); moreover, 5–20 % of the patients with lupus nephritis progress to ESRD within 10 years of disease onset (2) despite advances in our approaches in the treatment of lupus nephritis. Thus, the understanding of the pathogenesis of lupus nephritis remains a major challenge in the care of patients with SLE.

1.1. Genetic Contributions in Human SLE

During the last six decades, extensive data have been accumulated, suggesting that both genetic and environmental factors play significant role in the pathogenesis of SLE. Twin studies have provided definitive evidence that genetics as well as environment play important roles in human SLE: the concordance rate of SLE in monozygotic twins is 24–58 %, whereas in dizygotic twins or siblings, the rate was 2–5 % (3). SLE also displays familial aggregation, as the risk of disease among siblings of SLE patients is about 29 times higher than that among the general population (4). Recent genome-wide association studies on lupus susceptibility genes have identified more than 30 candidate genes that have relative minor impacts on SLE (5–8). Evidence has also been obtained showing ethnic variation in lupus clinical manifestations and the effects of social-economic factors on this disease (9). These data suggest the complexity of lupus genetics. The heterogeneity of the human population is still a major obstacle in genetic studies in man. It is also evident that to delineate the pathogenetic factors in a complex disease with protean clinical presentations present a formidable challenge. Studies on mouse models on lupus in general and lupus nephritis in particular have helped significantly in our understanding of this disorder. They will remain useful complementing human studies (10, 11). In this chapter, the genetic approaches in mapping genes contributing to lupus nephritis in mouse are reviewed briefly. Our studies on NZM2328 are discussed in detail to illustrate the methods for mapping genes that contribute to the development of lupus nephritis in mouse.

1.2. Mouse Models of Spontaneous Lupus Nephritis

New Zealand mice were the first mouse model described for human lupus (*reviewed in* (12, 13)). (NZB $\times$ NZW) F_1 females develop glomerulonephritis (GN) that resembles human proliferative lupus nephritis with immune complex deposition. New Zealand mice have been studied extensively. Subsequently, two other models, MLR/*lpr* and BXSB have been described. Although these three mouse models have important similarities, they differ significantly in their natural history, autoantibody profiles, organ involvement, and sex predilection (11, 14). It was recognized early that the background genes influence phenotypic expression. Thus, MRL/*lpr* has severe autoimmune phenotypes with autoantibody production, vacuities, skin disease, and severe GN while B6.*lpr* has very few autoimmune traits. During the past two decades, recombinant inbred strains have been established from a (NZB $\times$ NZW) F_2 or (NZB $\times$ NZW) F_1 $\times$ NZW mating (15). These strains have approximately 75 % of NZW with 25 % NZB genes. They have ANA and varying degree of GN (16). These strains are very useful for mapping genes contributing to lupus GN.

The aforementioned spontaneous lupus-prone models have been used in mapping genes contributing to various autoimmune phenotypes (*reviewed in* (10, 11, 17–19)). Typically, genetic analysis was carried out to identify genetic segments with quantitative trait loci (QTL) that contribute specifically to particular autoimmune phenotypes as distinguished by a quantitative trait by the study of either a cohort of (lupus-prone strain $\times$ non-lupus prone strain) F_1 backcrossed to the non-lupus strain or a cohort of inter-cross (F_2) progenies. The identified QTL needs to be substantiated by the generation of congenic strains in which the genomic region corresponding to the QTL of interest is introgressed to the non-autoimmune strain. This approach was taken by Wakeland, Morel and colleagues in their studies of *Sle1*, *Sle2*, and *Sle3*. In this case, *Sle1*, *Sle2*, and *Sle3* on chromosome 1, 4, and 7, respectively, were identified to be linked to GN by the analysis of a cohort of (NZM2410 $\times$ C57BL/6) F_1 $\times$ C57BL/6, in which NZM2410 is a lupus-prone strain while C57BL/6 (B6) is the non-lupus prone strain. Although autoantibody production was one of the traits of interest, no such QTL was identified. None of the congenics, B6.*Sle1*, B6.*Sle2*, and B6.*Sle3* have GN; however, the tricongenic B6. *Sle1 Sle2 Sle3* has severe GN. Although the functions of these

three loci in B6-based lupus congenics have been studied extensively, the nature of the genes conferring the GN phenotypes remains elusive (*reviewed in* (11, 19)).

As an alternative approach, we have chosen NZM2328 as the lupus-prone strain and C57L as the non-lupus strain. As detailed below, we have identified a single locus *Cgnz1* controlling chronic GN on chromosome 1, a single locus *Adnz1* on chromosome 4 linked to ANA and anti-dsDNA production, and three loci, *Agnz1* on chromosome 1, the H-2 complex and a locus, *Agnz2*, distal to the H-2 complex on chromosome 17 to be linked to acute GN. The phenotypes of NZM2328-base congenic strains, NZM2328.Lc1 and NZM2328.Lc4 confirm the genetic data (20, 21).

These two contrasting approaches underline the importance of the choice of strains and the defining of traits in the mapping of genetic traits for lupus nephritis.

1.3. NZM2328 as an SLE Model

NZM2328 female mice spontaneously develop severe proteinuria and lupus nephritis, which leads to early mortality. In contrast, male mice have ANA and anti-dsDNA antibodies and immune complex deposit in the kidney without severe proteinuria and early mortality. Thus, the NZM2328 strain displays a gender bias towards females, which is a feature of human SLE. Regarding autoantibodies, NZM2328 mice have ANA and anti-dsDNA antibodies; however, there are little anti-IgG antibody (rheumatoid factor) activities in the sera of NZM2328 mice. NZM2328 mice also have hypergammaglobulinemia (20). It was noted that severe proteinuria is correlated with the development of chronic GN and early mortality. Thus, severe proteinuria has been used as a biomarker for chronic GN and early mortality.

During the characterization of NZM2328, it was noted that two distinct stages of GN can be discerned in NZM2328: acute GN and chronic GN. Acute GN is marked by active proliferative GN, including signs of mesangial proliferation and glomerular hypercellularity without global sclerosis and tubular abnormality. In contrast, chronic GN is characterized by fibrotic changes, such as glomerulosclerosis, tubular atrophy and dilation, and interstitial fibrosis. In NZM2328 mice, lupus nephritis starts with acute inflammation primarily within the glomeruli, and then gradually progresses to the chronic form involving permanent damage in both the glomeruli and the interstitium (20). Between the stages of acute and chronic GN, we have documented a transitional disease phase in NZM2328 mice, which is histologically distinctive (22). Interestingly, NZM2328 males develop acute GN without progression to end-stage renal failure by 12 months of age (20). However, the mechanisms controlling the progression of lupus nephritis remain to be determined.

1.4. Systemic Lupus Nephritis Loci in NZM2328

To identify genes that contribute to the development of lupus nephritis, we conducted a genome-wide linkage analysis using a backcross cohort derived by crossing SLE-prone NZM2328 and non-lupus C57L mice. The backcross offspring were PCR genotyped with a genome-wide panel of simple sequence length polymorphism (SSLP) markers; they were also characterized in terms of lupus traits. SLE trait analysis involved following individual offspring until 12 months of age or when severe proteinuria developed, whichever occurred first. As stated above, QTL for acute and chronic GN (SLE traits) were genetically mapped; acute GN QTLs *Agnz1* and *Agnz2* were positioned on chromosomes 1 and 17, respectively, and a chronic GN QTL *Cgnz1* was mapped on distal chromosome 1. In addition, a single locus, *Adnz1* was located on chromosome 4 (20). Because *Cgnz1* and *Adnz1* were monogenic for association with ANA/anti-dsDNA and anti-nucleosome antibody production and chronic GN, respectively, congenic lines, NZM2328.Lc1 and NZM2328.Lc4 were

generated by introgressing the genetic regions containing these two loci into NZM2328. These congenic lines have been informative and are discussed separately (21).

1.5. Independent Genetic Control of ANA/Anti-dsDNA and Anti-nucleosome Antibody Production and GN

A cohort of NZM2328.Lc1 female mice were monitored over a period of 12 months for the production of ANA/ANA/anti-dsDNA and anti-nucleosome antibody production and the development of chronic GN. The results showed that these females did not produce ANA, anti-dsDNA, and anti-nucleosome antibodies and confirmed the genetic analysis. Despite the lack of production of these antibodies, these mice had severe proteinuria and chronic GN. The kinetics of development of severe proteinuria and chronic GN was similar to that of the parental strain NZM2328. These results demonstrate that ANA and anti-dsDNA and anti-nucleosome antibodies are not needed for the development of GN. They suggest that the hypothesis that breaking tolerance to these autoantigens is the first and crucial step in lupus pathogenesis (19) should be modified. Thus, a broader view regarding autoantibody role in lupus nephritis is advocated (23).

1.6. *Cgnz1* and *Agnz1* Verification and SLE-Gene Precision Mapping on Chromosome 1

The findings of *Cgnz1* and *Agnz1* agree with previous genetic studies on murine SLE, as the *Cgnz1* and *Agnz1* interval overlap with *Sle1* and *Sle1* was found to be linked to GN in a genome-wide linkage analysis of a (NZM2410 × C57BL/6)F₁ × NZM2410 backcross cohort (20).

To verify the biological function of the *Cgnz1* and *Agnz1* loci, we generated NZM2328.C57Lc1 (Lc1) congenic mice by introgressing a 24-cM chromosome 1 fragment of C57L/J, which spans *Cgnz1* and *Agnz1* loci, onto the NZM2328 background. The Lc1 mice had significantly reduced incidences of both acute and chronic GN, autoantibody production, severe proteinuria, and early mortality (21); these observations were consistent with our linkage findings (20). The successful generation of this important congenic model to study lupus GN in animals with disparate chromosome 1 intervals was largely due to the congenic approach we employed (i.e., using NZM2328 as the recipient background instead of a non-lupus strain). On the other hand, GN cannot be directly examined in congenic strains produced by the opposite approach as discussed and detailed in Subheading 3.2.

We have generated multiple intrachromosomal recombinant strains of NZM2328.Lc1 by monitoring cohorts of (NZM2328 × NZM2328.Lc1)F₂ for recombinations. Preliminary data indicate that replacing the regions of *Agnz1* had no effect on the development of both acute and chronic GN and the replacement of the regions controlling chronic GN inhibited the development of chronic GN despite the manifestation of acute GN with immune complex deposition (24). Furthermore, the region controlling chronic GN has been mapped to a 1.34 Mb segment of chromosome 1 (22). This paves the way for the identification of the gene controlling chronic GN by transgenesis or by knock-in approach. This aspect of mapping the genes for lupus nephritis is not discussed further in this chapter.

2. Materials

1. Gentra Puregene Mouse Tail Kit (QIAGEN, Valencia, CA).
2. Isopropanol.
3. 70 % ethanol.
4. 10× PCR buffer (without MgCl₂) (Applied Biosystems, Foster City, CA).

5. Primers for microsatellite markers with or without fluorescence labeling (Integrated DNA Technologies, Coralville, IA).
6. 25 mM MgCl₂.
7. 2.5 mM dNTPs: 2.5 mM dATP, 2.5 mM dTTP, 2.5 mM dCTP, 2.5 mM dGTP (Invitrogen, Carlsbad, CA).
8. 5 U/μl Taq DNA polymerase (Applied Biosystems).
9. MetaPhor agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME).
10. 1× TBE buffer (pH 8.0): 89 mM Tris, 89 mM Boric Acid, and 2 mM EDTA (pH 8.0).
11. Ethidium bromide.
12. 6× gel loading dye containing Bromophenol Blue (New England BioLabs, Ipswich, MA).
13. 50-bp DNA ladder (New England BioLabs, Ipswich, MA).
14. Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems).
15. MicroAmpTM Optical 96-Well Reaction Plate (Applied Biosystems).
16. Genescan 400HD Rox Standard (Applied Biosystems).
17. GeneMapper Software (Applied Biosystems).
18. Hi-Di Formamide (Applied Biosystems).
19. QTL mapping software.

3. Methods

The methods described here outline (a) classical genetic analysis and quantitative trait mapping in backcross mice; (b) congenic strain production; (c) precision genetic mapping for lupus nephritis loci; and (d) histological evaluation of GN. In addition to providing these protocols, we will utilize our work on NZM2328 to emphasize important considerations in carrying out mapping studies for SLE susceptibility genes.

3.1. Classical Genetic Analysis and SLE Trait Mapping in NZM2328 Backcross Mice

3.1.1. Generation of NZM2328 Backcross Mice and SLE Trait Development

1. Breed lupus-prone NZM2328 with non-lupus C57L/J mice to generate (NZM2328 × C57L/J)_{F1} progeny (see Note 1 for discussion of mating direction). _{F1} males and females should be kept for further backcrossing and phenotype analysis. All _{F1} pedigree records (e.g., ID of breeding cage and ID of both parents) must be maintained for future reference.
2. Breed NZM2328 with (NZM2328 × C57L/J)_{F1} mice to generate [(NZM2328 × C57L/J)_{F1} × NZM2328] backcross offspring (and see Note 1). Alternatively, use a

¹Whether to breed NZM2328 males or females with C57L/J for the _{F1} progeny is a practical choice that depends on reproductive ability and availability of males and females of both NZM2328 and C57L/J. In our experience, NZM2328 females are poor breeders, whereas NZM2328 males breed well even above 6 months of age.

For the breeding of backcross mice, use _{F1} females for mating if the QTL could be X-linked. However, if the goal is simply to map an autosomal mutation, the direction of the backcross can be chosen arbitrarily. Since _{F1} female mice tend to be very prolific, it is preferable to use them for the backcrossing.

In cases where the possibility of X-chromosome-linkage has not been excluded previously, it is wise to carefully pedigree all _{F1} and subsequent matings and progeny, in case genetic heterogeneity is encountered. In addition, both male and female _{F1} mice should be phenotyped to assess whether X-chromosome-linkage exists for the traits under investigation.

brother $\times$ sister (NZM2328 $\times$ C57L/J) F_1 intercross mating scheme to generate F_2 offspring for genetic analysis (see Note 2).

3. Monitor SLE traits (i.e., severe proteinuria and kidney histology) in NZM2328 backcross females over the period of 12 months after birth (see Note 3).

3.1.2. Extraction of Mouse Tail Genomic DNA

1. Clip 2–3 mm tail tissue from control NZM2328 and C57L mice and backcross offspring and store in a microcentrifuge tube (1.5 ml) labeled with the mouse ID at -20°C .
2. Add 300 μl Cell Lysis Solution (Gentra) and 1.5 μl Puregene Proteinase K (final conc. 0.1 mg/ml) to mouse tail tissue (fresh or frozen) and mix gently by inverting 25 times.
3. Incubate at 55°C overnight or until the tissue has completely dissolved. Invert tube periodically during the incubation. When tail tissue is not completely dissolved after overnight digestion, additional Proteinase K may be added, followed by 1–2 h further incubation at 55°C .
4. To quickly cool the tail lysate, incubate on ice for 1 min. Add 100 μl Protein Precipitation Solution (Gentra), and mix well by vortexing vigorously for 20 s at high speed.
5. Centrifuge sample at $\sim 16,000 \times g$ for 3 min at room temperature. The protein precipitate should form a tight pellet. However, if the pellet is loose, incubate on ice for 5 min and repeat the centrifugation.
6. In a clean microcentrifuge tube, add 300 μl isopropanol. Carefully decant the supernatant from step 5 into the tube with isopropanol. Note that the protein pellet should not be dislodged during pouring.
7. Mix sample gently by inverting 50 times, centrifuge sample at $\sim 16,000 \times g$ for 1 min at room temperature. The DNA precipitate may form a visible small white pellet. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper. Be careful not to lose the DNA pellet during the process, as it might be loose and easily dislodged.
8. Add 300 μl of 70 % ethanol and mix gently by inverting several times to wash the DNA pellet.
9. Centrifuge sample at $\sim 16,000 \times g$ for 1 min. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper. Be careful not to lose the DNA pellet.
10. Air dry the DNA pellet for 10 min. Add 50 μl DNA Hydration Solution (Gentra) and vortex 5 s at medium speed to mix. Incubate tail DNA samples at room temperature overnight to allow DNA rehydration. The DNA can be safely stored at $4-8^\circ\text{C}$ (short-term) and at -20 or -80°C (long-term).

²In general, if there is evidence of directional dominance (e.g., a particular phenotype in one strain caused by a combination of two or more recessive factors), it is preferable to backcross F_1 mice to the recessive parental strain. On the other hand, the intercross design provides better precision in mapping, and is more powerful for detecting QTLs that lack dominance (i.e., when heterozygotes have intermediate phenotype between the two homozygous parental strains). Therefore, when little can be assumed about the genetic control of a trait of interest, the intercross design is preferred.

³We selected only female backcross mice for the linkage analysis due to the gender bias toward NZM2328 females in the development of severe proteinuria and fatal GN (20). The required number of backcross mice depends on the strength of the QTL effect. In addition, time and cost are other factors to be considered. See reference (25) for guidance in determining useful cohort sizes.

3.1.3. Simple Sequence Length Polymorphism and Single Nucleotide Polymorphism Marker Selection for Genome-Wide Linkage Analysis—Detection of large and small effect QTLs in a genome-wide linkage analysis requires genetic markers spaced at ~20-cM distances, and no more than 10-cM from chromosome ends, for all chromosomes in the genome (25). Microsatellite repeat sequences in the mouse genome work well for the purpose of generating SSLP markers to readily distinguish allele variants in common laboratory strains of mice, including NZB and NZW (20, 26). SNP markers to distinguish NZB and NZW mouse alleles have been published (20, 26).

1. Published SSLP (also referred to as PCR marker) and SNP markers for 89 strains of mice may be obtained through the Mouse Genome Informatics (MGI), which can be accessed at <http://www.informatics.jax.org/>. Detailed marker information includes mouse chromosome position, product (allele) size and amplification primers (PCR markers), and nucleotide differences for SNP markers.
2. When strain-specific allele information is not available in public databases for potentially informative and useful SSLP and SNP markers based on their chromosome location, these must be tested against the control progenitor strains of interest using the methods described in Subheadings 3.1.4 and 3.1.5.
3. In practice, one should assemble and test the entire panel of select genetic markers, which can readily distinguish SSLP and SNP allele differences in select progenitor strains and their hybrid offspring. We have routinely used SSLP markers to reliably and accurately genotype alleles distinguished by 2+ bp differences in backcross and intercross progenies.
4. All hybrid offspring must be genotyped using the approved panel of genetic markers.

3.1.4. PCR Genotyping Mouse Tail DNA with SSLP Markers—Mouse tail DNA genotyping by PCR is carried out with select SSLP markers as discussed in Subheading 3.1.3.

1. Prepare PCR master mix sans tail DNA based on number of mouse tail DNA samples to be genotyped per SSLP marker.
2. Aliquot PCR master mix into individual PCR tubes, add tail DNA, and carry out PCR on a thermal cycler according to the conditions given in step 3.

Component	Volume per reaction (μl)	Final concentration
10× PCR buffer (No MgCl ₂)	1.0	1×
25 mM MgCl ₂	1.0	2.5 mM
2.5 mM dNTPs	0.8	0.2 mM
2.5 μM forward primer	1.0	0.25 μM
2.5 μM reverse primer	1.0	0.25 μM
ddH ₂ O	4.15	
5 U/μl Taq DNA polymerase	0.05	0.25 U/10 μl
Mouse Tail DNA	1.0	20–50 ng/10 μl
Total volume	10 μl	

3. Amplify SSLP Markers using the following PCR conditions.

Initial denature	94 °C for 3 min
PCR cycles	30 cycles of:
	94 °C for 20 s
	55 °C for 30 s
	72 °C for 30 s
	72 °C for 3 min
Final extension	72 °C for 3 min

3.1.5. Size Analysis of PCR-Amplified SSLP Markers—Two methods to separate and visualize SSLP PCR-amplified markers are detailed here.

Capillary Polymer-Based SSLP Size Analysis: Electrophoretic mobility in polymer-filled capillaries offers a convenient method to resolve PCR-amplified SSLP marker size differences. In this protocol, PCR is run with a fluorescent-labeled (5') primer so that SSLP markers, which have incorporated a fluorescent label, can be excited by an argon laser and visualized in the Genetic Analyzer 3130xl (Applied Biosystems). Compatible fluorescent dyes for PCR primers include 6-carboxyfluorescein (6-FAM, blue), hexachlorofluorescein phosphoramidite (HEX, green), NED (yellow), and ROX (red). The Genetic Analyzer can separate a complex mixture of DNA fragments (labeled with different fluorescence dyes) in one sample and determine the length of each fragment. Thus, these dyes can be used in multiplex PCR. SSLP marker mobility in polymer-filled capillaries is compared against ROX-labeled sized standards to determine allele sizes. This method automates marker calls and yields outstanding run-to-run consistency and reliability.

1. Run SSLP marker PCR according to Subheading 3.1.4 with one of the primers (forward or reverse) 5'-labeled with a compatible dye. Note that SSLP markers can be multiplexed in PCR with judicious selection and optimization of particular SSLP markers based on size and/or 5'-primer label differences and when PCR primers for the different markers do not form primer dimers.
2. Quickly spin down PCR products. Transfer 1 µl of each PCR product to a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems).
3. Prepare size standard by diluting Genescan 400HD ROX Size Standard (Applied Biosystems) 1:40 in Hi-Di Formamide (Applied Biosystems). Mix well by vortexing.
4. Add 9 µl of the ROX/Formamide mixture to each PCR product in the reaction plate from step 2, seal the plate with a septa (Applied Biosystems), and mix well by vortexing.
5. Quickly spin down the reaction plate, denature samples in the reaction plate on a thermal cycler at 95 °C for 5 min, and then immediately place the reaction plate with PCR samples on ice to incubate for 5 min.
6. Load the reaction plate with denatured PCR samples onto the Genetic Analyzer 3130xl and run samples per manufacturer's instruction.
7. Analyze the electrophoretic mobilities of SSLP markers with Data Collection (v3.0) and GeneMapper Software (Applied Biosystems) according to the manufacturer's instructions.

MetaPhor Agarose Gel Electrophoresis-Based SSLP Size Analysis: MetaPhor agarose (Cambrex Bio Science Rockland, Inc., Rockland, Maine) has an intermediate melting temperature of 75 °C and offers outstanding resolving capacity for separation of PCR-amplified SSLP markers which differ by 5+ bp. To insure accurate genotypes, we routinely run SSLP PCR with progenitor strain (i.e., NZM2328 and C57L/J) controls on the same MetaPhor gel with all offspring PCR products under analysis (27). To achieve optimum resolution, users must adhere strictly to manufacturer's instructions when preparing MetaPhor agarose gels.

1. Add sufficient quantity of MetaPhor agarose slowly to chilled 1× TBE buffer in a beaker with continuous swirling to make a 2 % solution.
2. After soaking agarose in cold 1× TBE buffer for ~15 min, weigh the beaker and solution, cover the beaker with plastic wrap with a small hole, and heat the beaker in a microwave oven until all the particles are dissolved.
3. Add sufficient heated distilled water (75 °C) to obtain the beaker and mix thoroughly. After cooling the solution to ~60 °C, add ethidium bromide (0.3 µg/ml), mix well, and cast the gel.
4. Allow gel to solidify at room temperature, and then condition it at 4 °C for 20 min before use. This maneuver is to insure optimal resolution and gel handling characteristics.
5. After adding 2 µl 6× gel loading dye to each 10 µl PCR product, load 10 µl of it on the MetaPhor gel submerged in chilled 1× TBE buffer and electrophorese for 3–4 h at 6 V/cm using horizontal electrophoresis system (see Note 4).
6. Visualize the PCR products under UV transilluminator and take photographs for records.
7. Make allelic calls by comparing sizes of PCR products of mice being genotyped to those of both parental strains.

3.1.6. Genetic Mapping of SLE Quantitative Trait Loci—To map chromosome locations for SLE QTL, NZM2328 back-cross genotypes and lupus trait data were analyzed using MapMangerQT (20). Other very useful QTL mapping programs include QTL Cartographer, R/qtl, and MapQTL (28, 29). Single-QTL genome scans using regression analysis typically detect chromosome map locations ranging from 10 to 30 cM based on likelihood ratio test statistic (LRS) or logarithm of the odds (LOD) scores. For a QTL, the mapping resolution depends on the number of recombination events in the mapping cohort. When assessing whether a QTL is statistically significant, Lander and Kruglyack's guidelines are standard practice: "Highly-significant" refers to $p < 0.001$, "significant" $p < 0.05$, and "suggestive" $p < 0.63$, after correcting for multiple testing in genome-wide scans (30). For a backcross genome-wide linkage analysis, the LOD score threshold for suggestive linkage is 1.9, and significant linkage 3.3 (30). When reporting QTL mapping results, the LOD scores, the peak positions, and the estimated confidence intervals should be provided. Once identified, one may further refine significant QTL locations by typing the initial backcross or intercross cohort with additional SSLP markers targeting these regions of interest, and then rerunning the QTL mapping program. Additionally, precision mapping for candidate loci may be carried out as described in Subheading 3.3.

⁴A 5-bp difference in PCR-amplified products (e.g., 95- and 100-bp SSLP markers) requires ~4 h to resolve by MetaPhor gel electrophoresis (27).

3.2. Generation of Non-SLE NZM2328 Congenic Strains

Following their identification in broad genome-wide linkage studies, SLE QTL must be verified at the biological level. A useful method to establish QTL validity is to produce a congenic strain by introgressing a donor chromosome fragment with its potential QTL onto a different genetic background which does not display the QTL effect. This may be accomplished via repeated backcrossing and genetic selection for donor alleles flanking a select QTL on the recipient strain background, but without donor alleles elsewhere in the genome.

3.2.1. Selection of Genetic Background for Congenic Strains—A critical first step toward precision SLE gene mapping and later functional studies is to select a desirable genetic background for the production of congenic strains. This is because background genetic factors may interact with or contribute to donor QTL allele effects, which may further influence whether a given QTL effect is manifest.

Recently, we produced NZM2328 chromosome 1-congenic mice with non-lupus alleles derived from C57L/J mice (21). An important feature in the NZM2328.C57Lc1 (also referred to as Lc1) mice is that only the select chromosome 1 interval is responsible for changes in disease progression normally observed in NZM2328 females. Thus, we can directly monitor the impact of *Agnz1* and *Cgnz1* on SLE traits in these mice. Inhibition of both acute and chronic GN was observed in Lc1 females, thus confirming that NZM2328 mice harbor one or more SLE QTL on chromosome 1 which contribute to disease (20).

In contrast to our approach, several related studies have used B6 mice, which do not develop lupus features, as recipients to generate congenic strains with donor alleles derived from lupus-prone mouse strains (e.g., NZM2410, NZW, and BXSB) (31, 32). An inherent weakness of the latter approach is that lupus traits have not manifested in the B6 background. For example, although *Sle1* and *Nba2* were initially mapped for GN, neither B6.*Sle1* nor B6.*Nba2* congenic strains developed GN. Instead, B6.*Sle1* mice developed increased levels of IgG ANA, and B6.*Nba2* congenic mice developed IgG anti-DNA and anti-chromatin autoantibodies (33, 34). Due to the lack of GN in B6.*Sle1*, the subsequent fine mapping of *Sle1* had to use autoantibody production as the trait to approximate GN (35). This approach has made Morel and colleagues to postulate the presence of *Sle1d* that confers the original phenotype of GN with unknown location within the *Sle1* region (35). Another weakness of the approach is that it potentially introduced a whole new set of gene interactions between non-lupus background and donor lupus alleles, complicating an already complex research question. In this regard, the non-lupus prone B6 has been shown to harbor a gene contributing to autoantibody production (36). Thus, we conclude that the introgression of non-lupus alleles into SLE-prone NZM2328 is highly useful and informative for the purpose of QTL verification and further study of acute and chronic GN and ESRD.

3.2.2. Speed Congenic Protocol—The traditional protocol for generating congenic strains is to back-cross a donor locus to a recipient genetic background for 10+ generations, accompanied by screening and selection for progeny carrying the donor locus at each generation. The founders are generated by intercrossing the mice of the last backcross and then selecting for homozygosity at the target region. Though straightforward, completion of this protocol takes 3–4 years (37). The marker-assisted selection protocol (MASP)-based breeding strategy, also known as the speed congenic approach, only requires 1.5–2 years (37). The basis of the speed congenic method is to select progeny at each backcross that carry the target donor region (or gene), but with minimal donor genetic contamination (i.e.,

heterozygosity) elsewhere in a recipient animal genome, which can dramatically accelerate completion of the desired congenic strain.

Another important consideration, when one generates congenic strains without phenotypic selection for the trait of interest, one must make certain that select donor alleles actually flank the potential QTL. In our experience, it is safer to start with a large chromosome segment to ensure that this will be the case, since it will be far simpler and less time-consuming to narrow the target region by generating subcongenic lines, than to generate a new congenic strain. The speed congenic protocol below describes how the chromosome 1 interval of NZM2328 carrying *Agnz1* and *Cgnz1* was replaced with a C57L-derived chromosome 1 fragment to produce the Lc1 congenic strain.

1. Produce 30–40 NZM2328 backcross (N_2) mice (15–20 mice of both sexes) as described in Subheading 3.1.1 (see Note 5).
2. Genotype NZM2328 backcross (N_2) mice with SSLP and SNP marker panel established in Subheading 3.1.2 to assess zygosity across the genome. SSLP markers for X and Y chromosomes were not included since a simple breeding step can fix the host sex chromosomes (see step 5).
3. Genotype NZM2328 backcross (N_2) mice with SSLP markers for the donor target region (i.e., the interval between markers *DIMit15* and *DIMit155*). Offspring were further screened with additional SSLP markers with ~5-cM spacing per marker to minimize the potential for intra-region recombination events. Select several NZM2328 backcross (N_2) mice that carry the donor allele(s) of interest and the highest percentage of recipient NZM2328 alleles elsewhere in the genome for further backcrossing.
4. Breed select NZM2328 backcross (N_2) mice with NZM2328 to produce 30–40 NZM2328 backcross (N_3) offspring and repeat step 2 with this new generation of mice.
5. Repeat steps 3 and 4 with NZM2328 backcross (N_3 – N_5) in place of N_2 backcross offspring until select NZM2328 back-cross (N_5) are obtained, which should contain the donor target region (heterozygous) in the homozygous NZM2328 background. Theoretically, donor genome contamination should be less than 0.5 % for N_5 backcross offspring by this method (37). To fix the sex chromosomes, make sure that at any one of the first three backcross generations (N_2 – N_4), female backcross mice were crossed with NZM2328 males, and that at the immediate following generation, male backcross mice were crossed with NZM2328 females.
6. Select a single pair of heterozygous NZM2328 backcross (N_5) mice that were from the same parents. Perform a complete genome-wide genotype scan on both animals to ensure that each retains the donor target region, but without donor genetic contamination elsewhere. Cross the pair to produce Lc1 homozygous offspring.
7. Genotype the offspring with the same set of informative SSLP markers. Select a breeding pair of animals that are homozygous for Lc1, and use them for all subsequent inbreeding (i.e., a brother $\times$ sister mating scheme) of Lc1 congenic mice.

⁵Genome-wide screening of 16 progeny per backcross generation with a low marker density (20–25 cM distance) is a cost-effective strategy (37).

3.3. Precision Mapping for SLE Genes on Chromosome 1

A major goal in precision mapping is to identify a minimal critical interval spanning the gene(s) responsible for a QTL effect, which is flanked or bounded by recombination crossovers. We have used this approach before to establish critical genomic regions needed in viral resistance, which subsequently allowed us to identify the relevant genes (38–43). More recently, we used this approach to pinpoint distinct locations for *Agnz1* and *Cgnz1* SLE loci within the larger (24-cM) chromosome 1 interval based on crossovers in a panel of Lc1 recombinant congenic lines (20, 22). Importantly, these results demonstrate that precision mapping is an important step in positional cloning and gene identification, even in narrow gene regions, based on findings obtained for three different locations in the mouse genome. As an example, the following method describes the generation of Lc1 recombinant congenic sublines.

1. Breed NZM2328.C57Lc1 mice, which are heterozygous for the Lc1 interval, in a brother x sister mating scheme. Most offspring produced in this breeding scheme will exhibit heterozygosity or homozygosity over the entire 24-cM Lc1 congenic interval. However, a fraction of the offspring will have a crossover on one or both chromosomes.
2. Genotype all offspring with select SSLP markers according to protocol in Subheading 3.1.4 to screen for recombination crossovers within the Lc1 congenic interval. Subheading 3.1.3 provides detailed information about how to obtain informative SSLP markers to distinguish alleles in the relevant strains.
 - a. When useful markers are unavailable, novel SSLP markers can be generated as we described previously (26, 42, 44).
 - b. In brief, download relevant genomic sequence covering the region of interest from the National Center for Biotechnology Information and manually inspect it for microsatellite repeat sequences.
 - c. Design sequence-specific primers to PCR amplify select microsatellite sequences.
 - d. Test SSLP marker primers to verify specific amplification of the select microsatellite, the capacity to distinguish the relevant alleles and genetic linkage (i.e., novel SSLP markers can be tested for linkage with other Lc1 markers in back-cross or intercross offspring DNA samples previously generated).
3. Examine Lc1 SSLP marker profiles for all offspring. Offspring, which display a potential recombinant crossover profile, must be verified with SSLP genotype analysis of newly prepared tissue DNA from the relevant animal.
4. Backcross the select and validated recombinant congenic mouse to NZM2328 to fix a new line. Repeat this step with each recombinant congenic animal to generate a mapping panel to precisely locate the QTL/gene(s) of interest.
5. Pinpoint recombination crossover boundaries with further SSLP marker PCR analysis to refine the size of the recombinant interval.
6. Monitor and record lupus features in the panel of recombinant congenic females over a period of 12 months.
7. Define *Agnz1* and *Cgnz1* critical regions based on recombination crossover locations and trait analyses.

3.4. Histological Scoring of Murine Glomerulonephritis

[(NZM2328 × C57L/J)F₁ X NZM2328] backcross mice were dissected at 12 months of age or at the time of being severely proteinuric for two consecutive weeks as determined by Multistix 10 SG (Bayer Diagnostics Division, Elkhart, Indiana). Signs of both acute and chronic renal pathology were scored separately by light microscopy.

3.4.1. Acute GN—Acute GN was scored blindly using a scale of 0–4, based on the severity of glomerular hypercellularity, mesangial matrix expansion, focal necrosis, and epithelial cell crescents (Fig. 1 B1, C1, D1, and E1). Grade 0 indicates the absence of signs of acute GN. Grade 1 indicates mild focal lesions with proliferative changes that are primarily in the mesangium. Grades 2 and 3 represent diffuse involvement of moderate severity in the glomeruli, whereas grade 4 indicates widespread changes of more severity (20).

3.4.2. Chronic GN—Chronic GN was scored blindly using a scale of 0–4, based on the severity of glomerulosclerosis (focal to global), tubular atrophy, dilated tubules with hyaline casts, and interstitial fibrosis (Fig. 1 B2, C2, D2, and E2). Grade 0 indicates no signs of chronic GN. Grade 1 represents mild focal glomerulosclerosis and interstitial inflammation. Grades 2 and 3 represent moderate glomerulosclerosis, tubule atrophy, and interstitial fibrosis. Grade 4 indicates severe glomerulosclerosis, tubule atrophy, and interstitial fibrosis (20).

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References

1. Cameron JS. Lupus nephritis. *J Am Soc Nephrol.* 1999; 10(2):413–424. [PubMed: 10215343]
2. Houssiau FA, Vasconcelos C, D'Cruz D, Sebastiani GD, de Ramon GE, et al. Early response to immunosuppressive therapy predicts good renal outcome in lupus nephritis: lessons from long-term followup of patients in the euro-lupus nephritis trial. *Arthritis Rheum.* 2004; 50(12):3934–3940. [PubMed: 15593207]
3. Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, et al. A revised estimate of twin concordance in systemic lupus erythematosus. *Arthritis Rheum.* 1992; 35(3):311–318. [PubMed: 1536669]
4. Alarcon-Segovia D, Alarcon-Riquelme ME, Cardiel MH, Caeiro F, Massardo L, et al. Familial aggregation of systemic lupus erythematosus, rheumatoid arthritis, and other auto-immune diseases in 1,177 lupus patients from the GLADEL cohort. *Arthritis Rheum.* 2005; 52(4):1138–1147. [PubMed: 15818688]
5. Deng Y, Tsao BP. Genetic susceptibility to systemic lupus erythematosus in the genomic era. *Nat Rev Rheumatol.* 2010; 6(12):683–692. [PubMed: 21060334]
6. Gualtierotti R, Biggioggero M, Penatti AE, Meroni PL. Updating on the pathogenesis of systemic lupus erythematosus. *Autoimmun Rev.* 2010; 10(1):3–7. [PubMed: 20863908]
7. Delgado-Vega A, Sanchez E, Lofgren S, Castillejo-Lopez C, Alarcon-Riquelme ME. Recent findings on genetics of systemic autoimmune diseases. *Curr Opin Immunol.* 2010; 22(6):698–705. [PubMed: 20933377]
8. Kaiser R, Criswell LA. Genetics research in systemic lupus erythematosus for clinicians: methodology, progress, and controversies. *Curr Opin Rheumatol.* 2010; 22(2):119–125. [PubMed: 20035223]
9. Borchers AT, Naguwa SM, Shoenfeld Y, Gershwin ME. The geoepidemiology of systemic lupus erythematosus. *Autoimmun Rev.* 2010; 9(5):A277–A287. [PubMed: 20036343]

10. Jorgensen TN, Gubbels MR, Kotzin BL. New insights into disease pathogenesis from mouse lupus genetics. *Curr Opin Immunol.* 2004; 16(6):787–793. [PubMed: 15511674]
11. Morel L. Genetics of SLE: evidence from mouse models. *Nat Rev Rheumatol.* 2010; 6(6):348–357. [PubMed: 20440287]
12. Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. *Adv Immunol.* 1985; 37:269–390. [PubMed: 3890479]
13. Borchers A, Ansari AA, Hsu T, Kono DH, Gershwin ME. The pathogenesis of autoimmunity in New Zealand mice. *Semin Arthritis Rheum.* 2000; 29(6):385–399. [PubMed: 10924025]
14. Andrews BS, Eisenberg RA, Theofilopoulos AN, Izui S, Wilson CB, et al. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *J Exp Med.* 1978; 148(5):1198–1215. [PubMed: 309911]
15. Rudofsky UH, Evans BD, Balaban SL, Mottironi VD, Gabrielsen AE. Differences in expression of lupus nephritis in New Zealand mixed H-2z homozygous inbred strains of mice derived from New Zealand black and New Zealand white mice. Origins and initial characterization. *Lab Invest.* 1993; 68(4):419–426. [PubMed: 8479150]
16. Rudofsky UH, Lawrence DA. New zealand mixed mice: a genetic systemic lupus erythematosus model for assessing environmental effects. *Environ Health Perspect.* 1999; 107(Suppl 5):713–721. [PubMed: 10502536]
17. Kono DH, Theofilopoulos AN. Genetics of SLE in mice. *Springer Semin Immunopathol.* 2006; 28(2):83–96. [PubMed: 16972052]
18. Theofilopoulos AN, Kono DH. Genetics of systemic autoimmunity and glomerulonephritis in mouse models of lupus. *Nephrol Dial Transplant.* 2001; 16(Suppl 6):65–67. [PubMed: 11568247]
19. Fairhurst AM, Wandstrat AE, Wakeland EK. Systemic lupus erythematosus: multiple immunological phenotypes in a complex genetic disease. *Adv Immunol.* 2006; 92:1–69. [PubMed: 17145301]
20. Waters ST, Fu SM, Gaskin F, Deshmukh US, Sung SS, et al. NZM2328: a new mouse model of systemic lupus erythematosus with unique genetic susceptibility loci. *Clin Immunol.* 2001; 100(3):372–383. [PubMed: 11513551]
21. Waters ST, McDuffie M, Bagavant H, Deshmukh US, Gaskin F, et al. Breaking tolerance to double stranded DNA, nucleosome, and other nuclear antigens is not required for the pathogenesis of lupus glomerulonephritis. *J Exp Med.* 2004; 199(2):255–264. [PubMed: 14718514]
22. Ge, Y.; Fu, SM. MS in preparation. In press
23. Bagavant H, Fu SM. New insights from murine lupus: disassociation of autoimmunity and end organ damage and the role of T cells. *Curr Opin Rheumatol.* 2005; 17(5):523–528. [PubMed: 16093828]
24. Ge Y, Jiang C, Gaskin F, Sung SJ, Bagavant H, et al. Pathogenesis of proliferative lupus nephritis: different genetic control for acute and chronic glomerulonephritis and new insight into the mechanism of immune complex mediated nephritis. *Arthritis Rheum.* 2009; 60:2019. [PubMed: 19565481]
25. Darvasi A. Experimental strategies for the genetic dissection of complex traits in animal models. *Nat Genet.* 1998; 18(1):19–24. [PubMed: 9425894]
26. Rodriguez MR, Lundgren A, Sabastian P, Li Q, Churchill G, et al. A Cmv2 QTL on chromosome X affects MCMV resistance in New Zealand male mice. *Mamm Genome.* 2009; 20(7):414–423. [PubMed: 19641964]
27. Asif M, Rahman M, Mirza II, Zafar Y. High resolution metaphor agarose gel electrophoresis for genotyping with microsatellite markers. *Pak J Agric Sci.* 2008; 45(1):75–79.
28. Manly KF, Olson JM. Overview of QTL mapping software and introduction to map manager QT. *Mamm Genome.* 1999; 10(4):327–334. [PubMed: 10087288]
29. Broman KW, Wu H, Sen S, Churchill GA. R/qtl: QTL mapping in experimental crosses. *Bioinformatics.* 2003; 19(7):889–890. [PubMed: 12724300]
30. Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet.* 1995; 11(3):241–247. [PubMed: 7581446]

31. Morel L, Rudofsky UH, Longmate JA, Schiffenbauer J, Wakeland EK. Polygenic control of susceptibility to murine systemic lupus erythematosus. *Immunity*. 1994; 1(3):219–229. [PubMed: 7889410]
32. Drake CG, Rozzo SJ, Hirschfeld HF, Smarnworawong NP, Palmer E, et al. Analysis of the new zealand black contribution to lupus-like renal disease. Multiple genes that operate in a threshold manner. *J Immunol*. 1995; 154(5):2441–2447. [PubMed: 7868910]
33. Mohan C, Alas E, Morel L, Yang P, Wakeland EK. Genetic dissection of SLE pathogenesis. Sle1 on murine chromosome 1 leads to a selective loss of tolerance to H2A/H2B/ DNA subnucleosomes. *J Clin Invest*. 1998; 101(6):1362–1372. [PubMed: 9502778]
34. Rozzo SJ, Allard JD, Choubey D, Vyse TJ, Izui S, et al. Evidence for an interferon-inducible gene, Ifi202, in the susceptibility to systemic lupus. *Immunity*. 2001; 15(3):435–443. [PubMed: 11567633]
35. Morel L, Blenman KR, Croker BP, Wakeland EK. The major murine systemic lupus erythematosus susceptibility locus, Sle1, is a cluster of functionally related genes. *Proc Natl Acad Sci U S A*. 2001; 98(4):1787–1792. [PubMed: 11172029]
36. Heidari Y, Fossati-Jimack L, Carlucci F, Walport MJ, Cook HT, et al. A lupus-susceptibility C57BL/6 locus on chromosome 3 (Sle18) contributes to autoantibody production in 129 mice. *Genes Immun*. 2009; 10(1):47–55. [PubMed: 18843275]
37. Wakeland E, Morel L, Achey K, Yui M, Longmate J. Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol Today*. 1997; 18(10):472–477. [PubMed: 9357138]
38. Scalzo AA, Brown MG, Chu DT, Heusel JW, Yokoyama WM, et al. Development of intra-natural killer complex (NKC) recombinant and congenic mouse strains for mapping and functional analysis of NK cell regulatory loci. *Immunogenetics*. 1999; 49(3):238–241. [PubMed: 9914339]
39. Scalzo AA, Wheat R, Dubbelde C, Stone L, Clark P, et al. Molecular genetic characterization of the distal NKC recombination hotspot and putative murine CMV resistance control locus. *Immunogenetics*. 2003; 55(6):370–378. [PubMed: 12920489]
40. Brown MG, Zhang J, Du Y, Stoll J, Yokoyama WM, et al. Localization on a physical map of the NKC-linked Cmv1 locus between Ly49b and the prp gene cluster on mouse chromosome 6. *J Immunol*. 1999; 163(4):1991–1999. [PubMed: 10438936]
41. Brown MG, Dokun AO, Heusel JW, Smith HR, Beckman DL, et al. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science*. 2001; 292(5518):934–937. [PubMed: 11340207]
42. Xie X, Stadnisky MD, Brown MG. MHC class I dk locus and Ly49G2+ NK cells confer H-2k resistance to murine cytomegalo-virus. *J Immunol*. 2009; 182(11):7163–7171. [PubMed: 19454713]
43. Xie X, Stadnisky MD, Coats ER, Ahmed Rahim MM, Lundgren A, et al. MHC class I D(k) expression in hematopoietic and nonhematopoietic cells confers natural killer cell resistance to murine cytomegalovirus. *Proc Natl Acad Sci U S A*. 2010; 107(19):8754–8759. [PubMed: 20421478]
44. Rodriguez M, Sabastian P, Clark P, Brown MG. Cmv1-independent antiviral role of NK cells revealed in murine cytomegalovirus-infected New Zealand white mice. *J Immunol*. 2004; 173(10):6312–6318. [PubMed: 15528370]

Fig. 1.

Stages of acute and chronic GN: Normal Kidney (**A**) from a 12-week-old NZM2328 female mouse. Kidneys from 24- to 28-week-old NZM2328 female mice with acute GN: (**B1**) stage 1 acute GN kidney showing mesangial expansion and some hypercellularity with normal tubular cells. Note that the glomerulus is bigger than that in normal kidneys, (**C1**) stage 2 acute GN showing more cellular infiltration in the glomeruli, (**D1**) stage 3 acute GN showing loci of interstitial infiltration with hypercellular glomerulus, and (**E1**) stage 4 acute GN showing segmental scleroses in the glomerulus without collapsing of the Bowman's capsule. The tubules are essentially normal. Kidneys from NZM2328 female mice older than 40 weeks with severe proteinuria and chronic GN: (**B2**) stage 1 chronic GN showing affected glomerulus with tubule dilatation and noncellular cast in the tubules, (**C2**) stage 2 chronic GN showing area of collapsed Bowman's capsule and a red cell cast in one of the tubules, (**D2**) stage 3 chronic GN showing generalized tubular dilatation and more extensive collapse of the Bowman's capsules, and (**E2**) stage 4 chronic GN showing sclerotic and necrotic glomerulus, with tubular dilatation and interstitial fibrosis. All photos were taken at 400× magnification.

Initial denature	94 °C for 3 min
PCR cycles	30 cycles of: 94 °C for 20 s 55 °C for 30 s 72 °C for 30 s 72 °C for 3 min
Final extension	72 °C for 3 min