Diabetic nephropathy (DN) is the most common cause of end-stage renal disease (11) and is associated with significantly increased mortality. In patients with type 1 diabetes, ~30% will manifest DN (13). A major characteristic of DN is albuminuria, and this feature is a predictor for progression toward renal failure. Reducing urine albumin is an important therapeutic goal for preventing decline in renal function (21).

Studies of familial aggregation, racial and ethnic comparisons, and linkage analysis have indicated a significant genetic component to DN (3, 8), and currently there are major human genome-wide studies underway to identify genetic loci in diabetic populations that confer susceptibility to DN (14). However, the identification of specific genes underlying DN in humans has proven difficult, expensive, and time consuming. This is due in part to the heterogeneity of the genome of human populations as well as uncontrolled environmental factors. Inbred strains of rodents are not encumbered by the difficulties of genetic heterogeneity or environmental variation. Since quantitative trait loci for many complex traits are concordant among mice, rats, and humans, genome scans in animal models are relevant to human diseases (17, 18) and can provide more rapid results.

The OVE26 (OVE) mouse carries a transgene overexpressing calmodulin in pancreatic β cells, resulting in early onset of type I diabetes (6). At present, it is the diabetic mouse line which manifests by far the most profound albuminuria (20, 25).

These mice have been maintained on an inbred FVB background. The purpose of the current study was to determine whether the FVB background was important to susceptibility to severe albuminuria in OVE mice. Our results showed that genetic background plays a surprisingly essential role. When OVE mice were bred with C57BL6 (C57) or DBA2 mice, profound albuminuria almost disappeared, although equivalent elevated blood glucose levels were still observed. Morphological studies indicated that offspring from OVE by C57 crosses had milder renal morphological changes coincident with reduced albuminuria. After crossing and backcrossing OVE and C57 mice, a genomic scan was conducted to identify possible genetic loci associated with diabetic albuminuria; one significant locus and three suggestive loci were found.

MATERIALS AND METHODS

Animals. Normal C57 and FVB mice were obtained from the National Cancer Institute (Frederick, MD). The type I diabetes mouse model OVE, which overexpresses calmodulin in pancreatic β cells (6), is bred in our laboratory on an FVB background. OVE mice on the background FVB are referred to as OVE. Two mating models were used to breed OVE-N2 (F2) progeny. One involved mating an OVE male with a C57 female to produce a positive male (OVE/C57F1), which was then backcrossed with an FVB female for OVE-N2 (F2) offspring; the other involved a cross between an FVB male and C57 female, with a resulting positive female (OVE/C57F1) backcrossed with a male OVE for OVE-N2 (F2) offspring. Diabetic females were not used for breeding since they have low fertility and they are poor mothers. All mice were maintained in a 12:12-h light-dark cycle with free access to water and food. All animal protocols were approved by the Animal Care and Use Committee at the University of Louisville.

Mouse streptozotocin diabetes models. Two-month-old male mice were administrated a single intraperitoneal dose of 200 mg/kg streptozotocin (STZ; Sigma-Aldrich) dissolved in 10 mM citric acid (pH = 4.5); 1 wk later, blood glucose was determined using a One Touch Glucose Meter (Johnson & Johnson). Mouse urine was collected at week 5 after STZ administration, and urine albumin was determined by ELISA, as stated below. For multiple low-dose (mld) STZ diabetes, 2-mo-old male mice were administrated intraperitoneal injections of 50 mg/kg STZ in 10 mM citric acid (pH = 4.5) every day for 5 consecutive days.

Urine albumin. Twenty-four-hour urine samples were collected from OVE/C57F1 and OVE-N2 mice at the age of 4.5 mo or from STZ-treated mice in individual metabolic cages (Nalgene, Braintree Scientific, Braintree, MA). To obtain sufficient urine volume, particularly from nondiabetic mice, a 10% liquid diet (Glucerna; Abbott Laboratories) was added to the feeding water, as previously described (25). Urine samples were centrifuged at 4,000 rpm for 10 min at 4°C and stored at −80°C. Urine albumin was determined using a Mouse Albumin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX) within the linear range of the assay as described by Zheng et al. (25). Some reports provide data as albumin excreted/24 h in diabetic and control animals, and other papers report the albumin/creatinine ratio. Because diabetes increases creatinine excretion in FVB mice, unlike other diabetic models (16), we used 24-h albumin excretion not normalized to creatinine for most assays in the FVB strain OVE mice.
For those assays normalized to creatinine, a QuantiChrom Creatinine Assay kit from Bioassay Systems (Hayward, CA) was used.

**Immunohistochemistry.** Kidney samples from FVB, OVE/C57F1, and OVE-N2 mice were cut in half sagittally and fixed for 18 h in 10% neutral buffered formalin solution (Surgipath Industry, Richmond, IL) and then transferred to 70% ethanol before being embedded in paraffin. The tissues were sectioned at 5 μm, and slides were prepared. Following deparaffinization and hydration, the sections were subjected to antigen retrieval using Dako target retrieval solution in a decloaking chamber (Biocare Medical). After blocking with normal serum, the sections were incubated with rabbit anti-mouse albumin antibody (1:500, Bethyl Laboratories) followed by Cy3-conjugated anti-rabbit antibody (1:100, Jackson Laboratories) for albumin detection (12).

**Renal histology.** Periodic acid-Schiff (PAS) staining for determination of mesangial matrix expansion was carried out as described by Zheng et al. (25). Images of 15 glomeruli/mouse, three mice/group were obtained from a single ×100 field, chosen if it contained at least 15 glomeruli. Images from this field were taken at ×400 magnification using a Nikon DS-Fil camera system connected to a Nikon E600 microscope. These images were then compared with a set of standard images in which no expansion was scored as 1, scores from 2 to 4 were based on standards with progressive expansion of PAS-stained matrix, and a score of 5 indicated the presence of Kimmelstiel-Wilson nodules. The same randomly ordered images were then blindly scored from 1 to 5 for the severity of matrix expansion, based on standard images. Mouse glomerular volume was calculated from at least 50 PAS-stained glomerular profiles from 3 mice/group as performed by Zheng et al. (25). Glomerular volume (VG) was calculated from the cross-sectional area with the formula $VG = \beta k(AG)^{1/2}$, where $\beta = 1.38$ is the shape coefficient for a sphere, and $k = 1.1$ is the size distribution coefficient.

Sirius red staining for kidney fibrosis was carried out as described by Grimm et al. (10). A semiquantitative scoring method was used to compare fibrosis between the groups. Random low-power ($\times 100$ final magnification) images of the Sirius red-stained sections were scored against standard images by a blind observer. The standard images covered the range of fibrosis staining observed in the samples and were scored 1, for minimal staining, to 3, for maximal staining.

**Serum glucose measurement.** Mouse blood samples were obtained from a tail tip cut using microhematocrit capillary tubes then centrifuged at 5,500 rpm × 10 min. Serum blood glucose was measured with an enzymatic glucose assay kit (Sigma-Aldrich) according to the manufacturer’s instructions.

**Genotyping.** Genomic DNA samples were obtained from mouse tails and isolated by a DNeasy Tissue Kit (Qiagen, Valencia, CA) with DNA purity confirmed by A260/280 ratio of 1.8–1.9. Mouse whole genome single nucleotide polymorphism (SNP) scans were performed using a high-resolution mouse 768 SNP panel at the Genetics Division and Harvard Partners Center for Genetics and Genomics at Harvard Medical School (Cambridge, MA) (7). This SNP panel was designed to optimize polymorphisms within C57 mice, and a total of 498 SNPs were informative between FVB and C57 inbred strains. The SNP marker density had an average distance of 3 cM between markers.

**Quantitative trait locus assay.** A mouse quantitative trait locus (QTL) assay was carried out by Iqtl software developed by Jackson Laboratory (Bar Harbor, ME). Albumin in 24-h urine samples was expressed in log_{10} of micrograms albumin excreted/24 h. Gender was set as an interactive factor since we have found diabetic female mice were more prone to albuminuria than male mice. A nonparametric test was used since data were not normally distributed as assessed by the Shapiro-Wilk normality test. The effective QTL linked with urine albumin were identified and expressed as the logarithm of the odds ratio (LOD) score. In this computation, the permutation number was set to 1,000, and $P = 0.05$ was set as the significant threshold for LOD (4).

**Statistical analysis.** All data are presented as means ± SE, with $P < 0.05$ indicating significant difference. Comparisons of urine albumin secretion and histology were analyzed by ANOVA or Student’s t-test depending on the number of groups.

**RESULTS**

**Urine albumin excretion in different diabetic mice.** As shown in Fig. 1, there is a large variation in published albuminuria for different diabetic models (15, 16, 19, 23, 25). Most of the diabetic models increased albuminuria <10-fold, and none of them reached over 50-fold except for the OVE model, which increased >200-fold compared with nondiabetic mice when expressed as 24-h urine albumin excretion. To evaluate OVE albuminuria when normalized to creatinine excretion, additional cohorts of OVE, normal FVB and C57 mice were tested (Supplementary Fig. S1). The fold-increase in albuminuria for OVE mice dropped to 98-fold when normalized to creatinine. The reduction was due to the greater creatinine output of OVE mice compared with normal FVB or C57 mice.

To examine a more commonly used background than FVB, we initiated crossing of OVE diabetic mice to C57 mice. In the F1 generation, designated OVE/C57F1, we found a dramatic drop in albuminuria, from >15,000 μg/24 h in OVE mice to ≈900 μg/24 h (Fig. 2A, $P < 0.001$). Similarly, breeding of OVE to the DBA2 strain reduced albuminuria to 471 ± 213 μg/day in the diabetic offspring ($n = 6$). The reduction in albuminuria does not appear to be a function of lesser diabetes, since there was no significant difference in serum glucose among OVE, OVE/C57F1, and OVE-N2 mice (Fig. 2B). To assess whether FVB mice had higher albuminuria than C57 mice for models of diabetes other than OVE, we produced diabetes with a single dose of 200 mg/kg STZ in male FVB, C57, and DBA2 mice. All tested STZ-treated mice had blood glucose values >600 mg/dl. Urine albumin measured after 5 wk showed that STZ diabetes produced four-fold higher albuminuria in FVB mice than in C57 or DBA2 mice (Fig. 2C, $P < 0.05$). Albuminuria after diabetes induced by mld treatment with STZ was not significantly higher in FVB mice than in C57 mice; however, there was a trend toward higher albuminuria in STZ A/J than in C57 or FVB mice.

**Urine albumin excretion in different diabetic mouse models.** Fold-increase in 24-h urine albumin or albumin-to-creatinine ratio induced by diabetes in different models compared with their nondiabetic counterparts is shown. OVE diabetes produced the greatest increase in albuminuria. eNOS, endothelial nitric oxide synthase.
mld STZ-treated FVB mice (P = 0.1). In our hands, DBA2 mice did not survive mld STZ treatment. For the purpose of direct comparison, 24-h albuminuria values of OVE mice on the FVB background, STZ-treated and normal FVB mice, and STZ-treated and normal C57 mice are combined on a single graph in Supplementary Fig. S2.

**Albumin staining.** We recently reported that in OVE mice on the FVB background, albumin accumulated in the lumen and epithelial cells of many tubules (12). This was assessed in the current study for normal FVB, OVE/C57F1, and OVE kidneys. In normal FVB and diabetic OVE/C57F1 kidney sections, albumin staining was hardly detected (Fig. 3, A and B). In contrast, OVE kidney sections showed many brightly stained albumin-positive tubules (Fig. 3C), consistent with our previous results (12).

**Glomerular matrix and glomerular volume expansion.** PAS staining (Fig. 4) was used to perform semiquantitative scoring of mesangial matrix, as described in METHODS. Compared with normal FVB mice, both diabetic groups showed expansion of glomerular matrix (Fig. 4, B and C). In addition, many diabetic glomeruli had acellular PAS-stained areas, and occasional diabetic glomeruli contained Kimmelstiel-Wilson nodules (Fig. 4C). However, as evident in the scoring (Fig. 4D), PAS staining revealed more mesangial matrix in OVE glomeruli than in OVE/C57F1 glomeruli (P < 0.01). Total glomerular volume displayed a similar trend (Fig. 4E). Both diabetic groups had larger glomeruli than normal FVB (P < 0.05), but OVE glomeruli were 63% larger than OVE/C57F1 glomeruli (P < 0.05).

**Fibrosis.** Sirius red staining was used to show renal interstitial fibrosis (Fig. 5). OVE kidney sections contained obvious areas of Sirius red staining around glomeruli and tubules (Fig. 5C). The staining was weaker in OVE/C57F1 mice and was rarely found in normal FVB sections (Fig. 5, A and B). As seen for glomerular pathology, both diabetic groups had more fibrosis than normal FVB (P < 0.05). Fibrosis scores in OVE sections was significantly worse than scores for OVE/C57F1 sections (P < 0.01).

**SNP whole genome scan and assay.** To determine whether there were identifiable genomic loci responsible for the strain
effects on albuminuria, DNA from 108 OVE-N2 offspring with accurate urine albumin data at 4.5 mo of age were scanned on a 768 SNP whole genome panel. Among the 108 backcross progeny, 57 were male and 51 were female. Urine albumin results were converted to log10. Male and female data were combined, and sex was set as an interactive factor in J/qtl software. Female data were normally distributed, but total male and total data were not; therefore, the analysis was done in nonparametric mode.

The assay result showed four peaks with LOD scores >2.6 (Fig. 6). The significant locus was centered at 76 cM on chromosome 11 [LOD = 2.98, P = 0.033 and 95% confidence interval (CI) from 63.35 to 83 cM]. Major but not quite significant loci were centered on chromosome 19 at 8.98 cM (LOD = 2.749, P = 0.056, 95% CI from 0 to 36 cM), chromosome 13 at 0.37 cM (LOD = 2.669 P = 0.073, 95% CI 0–8.5 cM), and chromosome 9 centered at 24.28 cM (LOD = 2.64, P = 0.074, 95% CI from 15 to 35 cM).

Fig. 3. Representative albumin staining (red) of kidney sections from FVB (A), OVE/C57F1 (B), and OVE diabetic mice (C). In FVB and OVE/C57F1 groups, there was minimal albumin staining; however, in OVE diabetic mice a large amount of albumin accumulated in many tubules (×20).

Fig. 4. Breeding OVE to C57BL6 reduces mesangial matrix expansion and glomerular volume. Representative periodic acid-Schiff (PAS) staining of FVB (A), OVE/C57F1 (B), and OVE (C) kidneys is shown. D: PAS staining scored by a blind observer, as described in MATERIALS AND METHODS. There were significant differences among all groups (*P < 0.001). E: average glomerular volumes calculated as described in MATERIALS AND METHODS. Scores and volumes were obtained from at least 45 randomly selected glomeruli obtained from 3 mice/group. All groups were significantly different (*P < 0.05).
Figure 7 shows the impact on albuminuria of homozygosity vs. heterozygosity for each of these loci. Homozygosity for the three loci on chromosomes 11, 13, and 19 had a positive effect on albumin excretion. This effect was greatest for chromosome 19: albuminuria was increased sevenfold for homozygous females and fourfold for homozygous males. Surprisingly, FVB homozygosity for the peak on chromosome 9 markedly reduced albuminuria. We also looked at the combined effect of the three positive peaks for albuminuria. As shown in Fig. 7E, combined homozygosity greatly enhanced the response observed for individual peaks: homozygous females were increased 10-fold and homozygous males were increased 30-fold, vs. heterozygous mice. For all individual loci and the combined loci, the effect of genotype on albuminuria was significant ($P < 0.02$ by 2-way ANOVA).

**DISCUSSION**

A limitation of experimental DN studies has been the lack of a mouse model with features of advanced human DN, including severe albuminuria. Compared with other models, OVE mice display much more profound albuminuria. We presumed that their extreme albuminuria was due to their severe, chronic hyperglycemia. However, in this study we found that a single cross of OVE mice to two other genetic backgrounds, DBA2 or C57BL6, reduced albuminuria 17-fold without significantly changing blood glucose. This demonstrates that factors other than the severity of hyperglycemia caused OVE albuminuria and that these factors were related to the background strain. Since crosses to two different strains decreased albuminuria it is likely that the severe albuminuria was due to susceptibility of the FVB strain to OVE diabetes, rather than unusual resistance of other strains.

We tested whether the susceptibility of FVB mice vs. C57BL6 mice applied to another model of diabetes, single high-dose STZ-induced diabetes. Here also, FVB diabetic mice had higher albuminuria, by more than fourfold, than C57BL6 or DBA2 mice. Breyer’s group (16) obtained similar results by mld STZ induction of diabetes: Their diabetic FVB mice had about five times higher 24-h albumin excretion than diabetic C57BL6 mice, and it was the highest of all strains tested. In the current study, following mld STZ induction of diabetes, FVB mice had higher albuminuria than C57 mice, but the difference did not reach significance. Considering STZ and OVE diabetes, it appears that FVB mice are one of the more sensitive strains for diabetes-induced albuminuria.
Histopathology of FVB OVE mice was worse than OVE/C57F1 mice with respect to interstitial fibrosis, mesangial matrix expansion, and glomerular size. While all of these differences were statistically significant, the magnitude of the strain effect on histology was small compared with its effect on albuminuria. Again, the difference cannot be due to the level of hyperglycemia. We suspect that the more severe pathology was secondary to the much greater albuminuria of FVB OVE mice. The effect of background strain on renal histopathology has previously been studied (16). Qi et al. (16) reported that mild STZ induced mesangial matrix expansion in FVB mice that was less than that of similarly treated DBA2 or C57BL6 mice. Glomerular basement membrane thickening in STZ-treated FVB mice was intermediate between DBA2 and C57BL6. Many prior studies (1), including our own on OVE mice (24), have indicated that increasing albuminuria aggravates histological pathology. We previously reported heterogeneous accumulation of albumin in FVB OVE proximal tubule cells (12), which we found in the current study did not occur in OVE/C57F1 tubules. The absence of albumin accumulation in low-albuminuria OVE/C57F1 mice supports our prior conclusion that albumin builds up in tubules only if albuminuria is severe.

A genome scan of DNA from 108 OVE-N2 offspring revealed 4 loci with LOD scores that were significant or nearly so. Our n value of 108 is relatively small, which may account for the failure of 3 large peaks to obtain significance at the 0.05 level. The peak with the greatest LOD score was at the 76 cM position of chromosome 11. We could find no reports of mouse loci near this position that contribute to renal pathology, nor could we find concordant loci for human or rat pathology. Thus this appears to be a newly identified locus for renal damage. A portion of the peak on chromosome 13 is orthologous to human region 7p13–15, which contains a locus previously associated with albuminuria (14). The chromosome 19 peak has a 95% CI from 0 to 36 cm, which overlaps the chromosome 19 QTL peak at 24 cm, previously linked with mouse albuminuria (5). The 95% CI of our mouse chromosome 19 peak corresponds to human genomic regions 9q21–q24, 10q11–q26, and 11q12–q13. There are several reports of loci associated with nephropathy in these regions. Human region 9q21 includes a locus associated with nephropathy in type 1 diabetics (14). Also, at 9q21 Arar et al. (2) found a significant locus associated with chronic kidney disease in Mexican Americans. A locus linked to albuminuria was found by Freedman et al. (9) at human chromosome 10q21.1 in type 2 diabetics. The peak we found on chromosome 9 is orthologous to human chromosome region 11q22–24, which contains a locus linked with FSGS (22). Surprisingly this locus on chromosome 9 reduced albuminuria when homozygous for the FVB genotype. In view of the strong opposite effect of the overall FVB genotype, this was unexpected and points to the complexity of genomic influence on albuminuria.

Each of the four individual genomic peaks had a statistically significant effect on albuminuria. For the three peaks that increased albuminuria on chromosomes 11, 13, and 19, the magnitude of the individual gene effect ranged from 2.5- to 5-fold. Of our 108 N2 mice, 16 were homozygous and 17 were heterozygous for all 3 of these loci. In this population of 33 mice, homozygosity resulted in a 12.5-fold increase in albuminuria. This is almost 75% of the difference in albuminuria we found between OVE and OVE/C57 mice. Therefore, we suggest that these three loci contain the major QTLs that confer susceptibility to diabetic albuminuria on the FVB strain of mice.

In conclusion, these results demonstrate that the very severe albuminuria of OVE mice is almost completely dependent on the inbred genetic background FVB. A single cross to DBA2 or C57BL6 reduces albuminuria ~17-fold. Coinciding with the drop in albuminuria, renal histopathology is significantly reduced. Evidence was obtained for four FVB loci that significantly affect OVE albuminuria and may prove useful in identifying genes that impact human DN.

GRANTS

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Fig. 7. Effect of zygosity on urine albumin excretion for the 4 LOD peaks on chromosomes 11 (A), 19 (B), 13 (C), and 9 (D). Homozygosity on all peaks except the peak on chromosome 9 increased urine albumin. E: combined effect of homozygosity or heterozygosity for the loci on chromosomes 11, 19, and 13. Results are from 108 OVE-N2 mice (A–D) and 33 mice (E). For all panels, albumin was significantly affected by genotype ($P \leq 0.02$ by 2-way ANOVA with sex and zygosity as factors).
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