

# Progressive glomerulosclerosis and enhanced renal accumulation of basement membrane components in mice transgenic for human immunodeficiency virus type 1 genes

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**ABSTRACT** Patients infected with human immunodeficiency virus type 1 (HIV-1) develop a renal syndrome characterized by proteinuria, renal failure, and focal segmental glomerulosclerosis. By using a noninfectious HIV-1 DNA construct lacking the *gag* and *pol* genes, three transgenic mouse lines have been generated that develop a syndrome remarkably similar to the human disease. In the present study, we have characterized in detail one of these lines, Tg26. In Tg26 mice, proteinuria was detectable at  $\approx 24$  days of age, followed by severe nephrotic syndrome and rapid progression to end-stage renal failure. Renal histology showed focal segmental glomerulosclerosis and microcystic tubular dilatation. Indirect immunofluorescence studies demonstrated increased accumulation of the basement membrane components laminin, collagen type IV, and heparan sulfate proteoglycan. The viral protein Rev was present in sclerotic glomeruli. Northern blot analysis of total renal RNA showed expression of viral genes prior to the appearance of histologic renal disease, with greatly diminished viral gene expression late in the disease course. Kidneys from transgenic mice expressed increased steady-state levels of collagen  $\alpha 1(\text{IV})$  mRNA when glomerulosclerosis was present. We conclude that the presence of HIV-1 genes is associated with progressive renal dysfunction and glomerulosclerosis in transgenic mice.

Among the protean manifestations now recognized as sequelae of infection with human immunodeficiency virus type 1 (HIV-1) is a renal syndrome, termed HIV-associated nephropathy. This syndrome is characterized clinically by heavy proteinuria, often with the nephrotic syndrome, and a high incidence of progression to end-stage renal disease (1–6). Pathologically, the most common glomerular lesions in HIV-associated nephropathy are focal segmental glomerulosclerosis and microcystic tubular dilatation (6). Some of the remaining patients have mesangial proliferation (7).

Recently, we developed transgenic mouse lines that bear HIV-1 genes. A defective form of the HIV-1 provirus that lacks 3 kilobases (kb) of sequence overlapping the *gag* and *pol* sequences was introduced into mice as a transgene. Transgenic lines derived from three founder animals developed proteinuria, progressive renal failure, glomerulosclerosis, and microcystic tubular dilatation. In the present study, we describe in detail the clinical, pathological, and molecular biological manifestations of the renal disease in one of these transgenic mouse lines (Tg26).

## MATERIALS AND METHODS

**Transgenic Mice.** The transgene, 7.4 kb long, consisted of the proviral HIV genome, lacking 3 kb of sequence overlapping the *gag* and *pol* genes. The remaining proviral sequence included the 5' and 3' long terminal repeats (LTRs) and the *env*, *tat*, *nef*, *rev*, *vif*, *vpr*, and *vpu* genes (8). The transgene was introduced into male pronuclei of FVB/N mouse oocytes, resulting in nine founder animals, all of which remained healthy. Male founder mice were back-crossed with normal female FVB/N mice to produce the F<sub>1</sub> generation. Three of the eight male founder animals (Tg22, Tg25, and Tg26) gave rise to lines that developed progressive renal disease in the F<sub>1</sub> generation, and all three of these founders had unique restriction fragment length polymorphisms for the transgene (9).

**Southern Blot Analysis.** Ten micrograms of tail DNA was digested with *EcoRI*, electrophoresed through 0.7% agarose gels, and transferred to nylon membranes. DNA was probed with a cDNA encoding the HIV-1 *nef* gene (10) radioactively labeled using [<sup>32</sup>P]dCTP (New England Nuclear/DuPont) by the random-primer method (Boehringer Mannheim). Hybridization was carried out at 37°C in 50% (vol/vol) formamide/dextran sulfate (0.1 g/ml)/5× SSPE/10 mM Tris-HCl, pH 7.5/4× Denhardt's solution/denatured salmon sperm DNA (0.1 mg/ml). (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA.) The filters were washed under highly stringent conditions at 68°C in 0.1× standard saline citrate (SSC)/0.1% SDS and autoradiographic exposures were made at -70°C.

**Serum and Urine Chemistry.** Blood (200  $\mu$ l) was obtained by retroorbital puncture at 20-day intervals. Blood urea nitrogen, serum cholesterol, and serum albumin were assayed using commercially available kits (Boehringer Mannheim). Urine protein was measured with a colorimetric assay using pyrogallol red/molybdate (Biotrol USA, West Chester, PA), and urine creatinine was measured by the picric acid method.

**Histology.** Zinc formalin-fixed paraffin-embedded renal sections were cut at 3  $\mu$ m and stained with periodic acid/Schiff reagent (PAS) or Yajima silver. Renal tissue was embedded in OCT compound (Tissue-Tek; Miles) and frozen in isopentane at -70°C. Sections (4  $\mu$ m) were cut, air-dried overnight at room temperature, and fixed in acetone at 4°C. Sections were blocked with normal goat serum diluted 1:5 in phosphate-buffered saline (PBS) and exposed to fluoresce-

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Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; PAS, periodic acid/Schiff reagent.

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inated primary antibody diluted 1:40 in PBS for 60 min at room temperature. Affinity-purified primary antibody included goat anti-mouse IgG, goat anti-mouse IgM, goat anti-mouse IgA (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and sheep anti-mouse complement (Bioscience International, Kennebunkport, ME).

**Immunohistology for Extracellular Matrix and Viral Proteins.** Paraffin sections (4  $\mu$ m) were deparaffinized and digested with 0.5% trypsin for 60 min at 37°C. Sections were blocked with normal goat serum, exposed to specific antibody diluted 1:40 in PBS for 60 min, and washed with PBS. Affinity-purified fluoresceinated goat anti-rabbit antibody (Kirkegaard and Perry), diluted 1:40 in PBS, was applied for 60 min. The specific rabbit antisera used included anti-mouse laminin and anti-mouse collagen type IV (kindly provided by Hynda Kleinman, National Institute of Dental Research), anti-mouse basement membrane heparan sulfate proteoglycan core protein (11), and antiserum directed against residues 26–51 of Rev.

**Extraction of Total RNA and Northern Blot Analysis.** Mouse kidneys were snap frozen in liquid nitrogen and total renal RNA was isolated by centrifugation through cesium chloride (12). RNA (10  $\mu$ g) was electrophoresed through 1% agarose/formaldehyde gels, transferred to nylon filters, and fixed by baking for 2 hr at 80°C. The following cDNA probes were <sup>32</sup>P-labeled using the random-primer method: a 1.2-kb *Hind*-III fragment of *nef* cDNA (10), a 1.5-kb *Eco*RI–*Hind*III fragment from mouse laminin B2 chain cDNA (13), a 0.8-kb *Pst*I–*Ava*I fragment of mouse collagen  $\alpha$ 1(IV) cDNA (14), and a human ribosomal S14 protein cDNA (American Type Culture Collection 59246). Hybridization was carried out at 37°C in 50% formamide/dextran sulfate (0.1 g/ml)/5 $\times$  SSC/10 mM Tris-HCl, pH 7.5/4 $\times$  Denhardt's solution/denatured salmon sperm DNA (0.1 mg/ml). The filters were washed at 55°C in 0.1 $\times$  SSC/0.1% SDS. Autoradiographic exposures were made at –70°C, and the blots were scanned on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA), which provided a direct measure of radioactivity.

**Statistics.** Data are expressed as mean  $\pm$  standard deviation. Student's *t* test was performed using the StatView II statistical package (Abacus Concepts, Berkeley, CA).

## RESULTS

F<sub>1</sub> offspring from the Tg26 founder were mated and the resulting litters contained nontransgenic homozygous (normal) mice, heterozygous transgenic mice, and homozygous transgenic mice, each with a distinct phenotype (Fig. 1A). Normal mice had full life spans and lacked histopathologic evidence of disease. Heterozygote transgenic mice were grossly indistinguishable from their normal littermates at birth but, subsequently, developed renal disease. Homozygous transgenic mice were runted at birth, had poor nutritional intake, and died between birth and 40 days of age. Southern blot analysis of tail DNA obtained from the homozygous transgenic mouse exhibited both a dominant 7-kb band (arrow) and fainter higher molecular weight bands (bars) as shown in Fig. 1B. The dominant 7-kb band represents multiple linked copies of the transgene and the fainter higher molecular weight bands represent flanking host DNA sequences adjoining the 5' and 3' regions of the viral genome. Southern blot analysis of tail DNA obtained from homozygous animals demonstrated twice the hybridization intensity of the heterozygotes.

Whereas all homozygous transgenic mice died shortly after birth, heterozygous mice developed severe edema between 60 and 250 days of age. On gross inspection, kidneys from these heterozygotes were found to be enlarged with microcystic changes. In addition to edema, these mice had hypoalbuminemia and marked hypercholesterolemia, other features

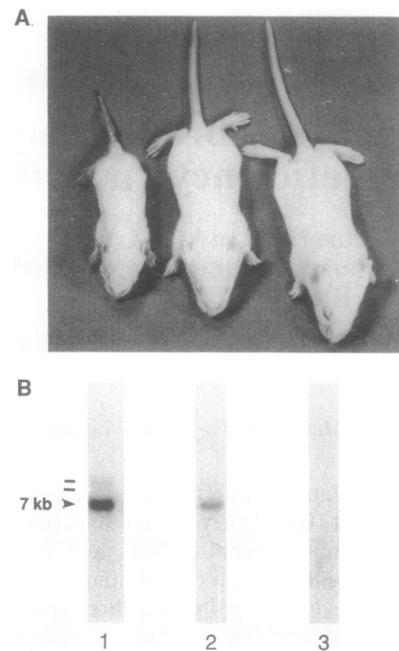


FIG. 1. Normal, heterozygous, and homozygous HIV-transgenic mice. (A) Breeding of heterozygous F<sub>1</sub> generation transgenic mice from line Tg26 produced offspring with three genotypes, shown here at 14 days of age: transgenic homozygous mice that were runted at the time of birth and generally died by age 30 days (*Left*), transgenic heterozygous mice that were phenotypically normal at this age (*Center*), and normal nontransgenic homozygous mice (*Right*). (B) Southern blot analysis was performed with tail DNA, cut with *Eco*RI, and probed with a cDNA encoding the *nef* gene. DNA from the transgenic homozygous mouse (lane 1) exhibited both a dominant 7-kb band (arrow) and fainter higher molecular weight bands (bars). DNA obtained from heterozygous animals had  $\approx$ 50% of the hybridization intensity as that of homozygous littermates (lane 2). Normal nontransgenic mouse DNA is indicated in lane 3.

of the nephrotic syndrome. In seven normal mice, serum albumin was  $3.5 \pm 0.5$  g/dl and serum cholesterol was  $143 \pm 26$  mg/dl, whereas in seven age-matched transgenic mice with edema, serum albumin was  $2.4 \pm 0.5$  g/dl ( $P < 0.01$ ) and serum cholesterol was  $387 \pm 148$  mg/dl ( $P < 0.001$ ). Death from uremia occurred within 10–15 days of the onset of edema.

To evaluate the course of the renal disease, we measured indices of renal function in a litter of mice longitudinally from birth. Urinary protein excretion, as measured by the ratio of urine protein concentration to urine creatinine concentration, was comparable in heterozygous transgenic mice and normal littermates at 19 days of age (Fig. 2A). At  $\approx$ 24 days of age, the transgenic mice developed proteinuria, which progressively increased with time. By 40 days of age, blood urea nitrogen was also significantly elevated in the heterozygous transgenic mice as compared to normal littermates (Fig. 2B). Among 203 heterozygous transgenic mice in 57 litters, 33 (16%) developed nephrotic syndrome and end-stage renal disease by 100 days of age and the remaining 170 transgenic mice had significant proteinuria. None of the normal 164 littermates have developed end-stage renal disease.

Kidneys harvested from transgenic animals at 45 days of age showed a spectrum of pathologic changes (Fig. 3). The renal tubules were notable for microcystic tubular dilatation (Fig. 3B) and atrophy and simplification of the tubular epithelium (Fig. 3D). Tubules were filled by densely staining PAS-positive material (Fig. 3B, E, and F). There was a sparse monocytic interstitial infiltrate and minimal interstitial fibrosis (Fig. 3D). Essentially all glomeruli manifested increased PAS-positive material, some with segmental (Fig. 3

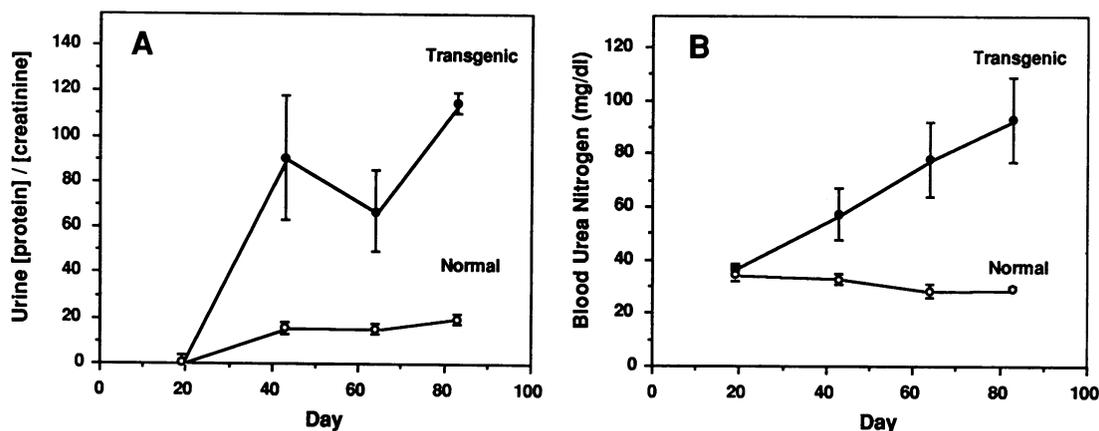


FIG. 2. Proteinuria and progressive renal failure in Tg26 mice. (A) Urine protein and creatinine were measured in heterozygous mice, and the ratio was calculated to give an estimate of protein excretion. Proteinuria appeared after day 20 and persisted until death. (B) Progressive azotemia was noted after day 40, and death from uremia occurred between days 60 and 200. In A and B, mean  $\pm$  standard deviation are presented, with open circles denoting normal littermates ( $n = 5$ ) and solid circles denoting transgenic mice ( $n = 4$  at 19 and 40 days,  $n = 3$  at 64 days, and  $n = 2$  at 83 days; all four transgenic mice developed nephrotic syndrome and died of end-stage renal disease at 64, 77, 85, or 85 days of age).

E and F) and others with global sclerosis (Fig. 3G). A minority of glomeruli showed mesangial hypercellularity (data not shown). Glomerular parietal and visceral epithelial cells appeared reactive, with enlargement and vacuolization (Fig. 3E and F). Glomerular synechiae were present but epithelial crescents were absent. Silver staining confirmed that the PAS-positive material within the glomerulus was composed of matrix components and demonstrated duplication of Bowman's capsule (Fig. 3H). Kidneys obtained from animals with severe renal failure exhibited diffuse global glomerulosclerosis and progressive tubular dilatation and atrophy (data not shown).

Immunohistology of kidneys from transgenic mice at 30–35 days of age revealed deposition of coarse granular aggregates of IgM, lesser amounts of IgA and C3, and, occasionally, IgG in a segmental distribution corresponding to the sclerotic regions of glomeruli (data not shown). Kidneys from normal littermates were devoid of immune deposits and complement. Indirect immunofluorescent analysis revealed accumulation of extracellular matrix proteins in the sclerotic regions of glomeruli from transgenic mice. Laminin, collagen type IV, and heparan sulfate proteoglycan were markedly increased in glomeruli from transgenic mice, with diffuse mesangial staining (Fig. 4B, D, and F). By contrast, Bowman's capsule, the tubular basement membranes, and interstitial regions of the kidneys of transgenic mice and normal littermates did not show differences in staining. Immunofluorescent studies demonstrated Rev protein in sclerotic glomeruli (Fig. 4H); similar studies with specific antisera directed against gp41, gp120, Tat, and Nef were negative (data not shown).

Northern blot analysis of total renal RNA was performed with a nef cDNA probe that overlaps the proviral 3' LTR and is, therefore, expected to hybridize with all proviral mRNAs (Fig. 5). Proviral mRNA of two size classes, 2 kb and 4 kb, was present at the earliest time points examined, 18 days (prior to the onset of proteinuria) and 28 days (after the onset of proteinuria but prior to the appearance of histologic changes in the kidney). By the time morphologic changes were well-established in the kidney, the expression of viral genes was severely attenuated (54 and 220 days).

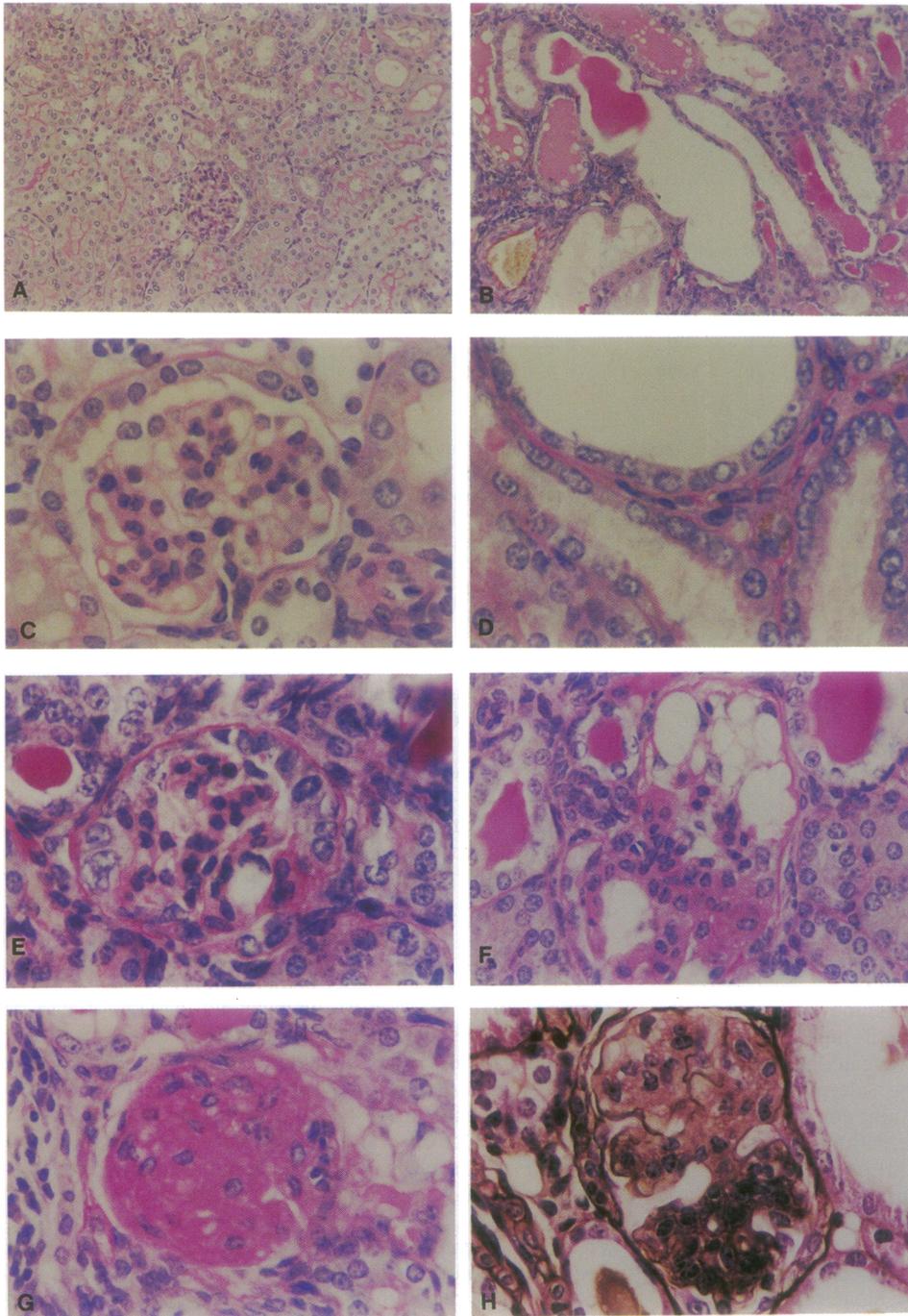
In the normal mouse kidney, levels of laminin B2 chain and collagen  $\alpha 1(IV)$  mRNA were highest at the earliest time point examined, 18 days, and declined with age, as has been reported (15). In the kidneys from transgenic mice, laminin B2 mRNA followed a quantitatively similar pattern. Transgenic kidneys expressed steady-state levels of collagen  $\alpha 1(IV)$  transcripts comparable to normal mouse kidneys at 18

and 28 days. In contrast, the steady-state levels of collagen  $\alpha 1(IV)$  mRNA were increased compared to normal littermates at day 54 and day 220. The ratio of collagen  $\alpha 1(IV)$  mRNA to S14 ribosomal protein mRNA was calculated to provide a normalized measure of steady-state collagen type IV mRNA expression. In normal mice, this ratio was 14.2 at 18 days, 2.4 at 28 days, 0.1 at 54 days, and 0 at 220 days, indicating no detectable collagen  $\alpha 1(IV)$  transcripts at the final time point. In the transgenic mouse, this ratio was 16.2 at 18 days, 2.9 at 28 days, 2.4 at 54 days, and 13.8 at 220 days, indicating increased steady-state amounts of collagen  $\alpha 1(IV)$  mRNA in older transgenic mice compared to normal mice.

## DISCUSSION

HIV-associated nephropathy is increasingly recognized as a complication of HIV-1 infection particularly in children (3, 5) and drug abusers (6). Despite the importance of this problem, the fundamental disease mechanisms remain to be elucidated. HIV-associated nephropathy may result from the presence of replicating virus within renal cells or from a direct or indirect effect of viral gene products acting upon target renal cells. It has been suggested that human glomerular cells express CD4 (16) and thus could be potential targets for HIV-1 infection. Alternatively, CD4-negative mesenchymal renal cells might be susceptible to infection, as has been demonstrated *in vitro* for other mesenchymal cell types that do not express detectable amounts of CD4 (17). It has been suggested that HIV-1 gene products may also cause disease in certain tissues directly. For example, Tat can be released by infected lymphocytes and can be taken up by uninfected target cells (18). Furthermore, the introduction of Tat into culture medium stimulates the growth of spindle cells isolated from Kaposi lesions from patients with AIDS (19) and mice transgenic for the *tat* gene under the control of the HIV-1 LTR develop mesenchymal tumors (20).

Although p24 core protein of HIV and genomic DNA sequences have been identified in both glomeruli and tubules from patients with HIV-associated nephropathy (21), it remains unclear whether replicating virus is present in the kidney and whether it is virus or viral protein products that are responsible for mesangial sclerosis. In the present study, the insertion of a construct with viral LTRs and encoding *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* genes was associated with the appearance of progressive glomerular and tubular abnormalities in heterozygous mice that closely resemble HIV-associated nephropathy. Thus, it appears that HIV-1 gene



**FIG. 3.** Renal histology in Tg26 mice. Low-power (PAS;  $\times 110$ ) views of normal mouse kidney (A) and HIV-transgenic mouse kidney (B), both aged 45 days, reveal microcystic tubular dilatation and a sparse monocytic infiltrate. At higher power, the glomerulus from the normal mouse is shown (C) (PAS;  $\times 450$ ). A high-magnification view of the tubulointerstitial region of 45-day-old transgenic mouse kidney demonstrates flattened atrophic epithelium from a dilated tubule, and an expanded interstitial compartment containing round mononuclear cells, spindle-shaped fibroblastic cells, and increased matrix components (D) (PAS;  $\times 450$ ). High-magnification views of 45-day-old transgenic mouse kidney show focal and segmental glomerulosclerosis (E and F) (PAS;  $\times 450$ ) and global glomerulosclerosis (G) (PAS;  $\times 450$ ). Silver stain demonstrates focal increase in mesangial matrix and thickening and wrinkling of Bowman's capsule in a 65-day-old transgenic mouse kidney (H) (Yajima stain;  $\times 450$ ).

products alone are capable of inducing many of the features of HIV-associated nephropathy.

The pathogenic mechanisms by which viral genes might produce glomerulosclerosis and tubular abnormalities in this model are unknown. Glomerular immune deposits appeared late in the disease course and did not appear to be specific. Thus, the glomerulosclerosis does not appear to be the result of immune-complex disease. Alternatively, viral gene products may be expressed in kidney or may be produced in peripheral tissues and accumulate in kidney. Viral gene products may be cytotoxic or may act upon renal parenchymal cells to stimulate extracellular matrix protein synthesis. The demonstration of Rev protein in sclerotic glomeruli would be consistent with either local production or nonspecific trapping of serum-derived material. The absence of other viral proteins in kidney may indicate that these genes are not transcribed or translated or that the proteins are present in quantities too small to be detected.

The expanded mesangial compartment was occupied by increased amounts of laminin, collagen type IV, and heparan sulfate proteoglycan. Collagen  $\alpha 1(\text{IV})$  mRNA was increased coincident with the appearance of glomerulosclerosis. This finding is consistent with either increased gene transcription or prolonged mRNA half-life, and these cannot be distinguished by the Northern blot analysis presented here. By contrast, laminin B2 mRNA was not substantially increased at any time point examined. Thus, it appears that the mechanisms that result in increased accumulation of basement membrane components in this model are complex and operate at both transcriptional and posttranscriptional levels.

Renal pathology in transgenic mice may be a consequence of mechanisms independent of the expression of the transgene itself, such as insertional inactivation leading to nephrotic syndrome and progressive glomerulosclerosis (22). Disruption or inactivation of host genes, however, is not

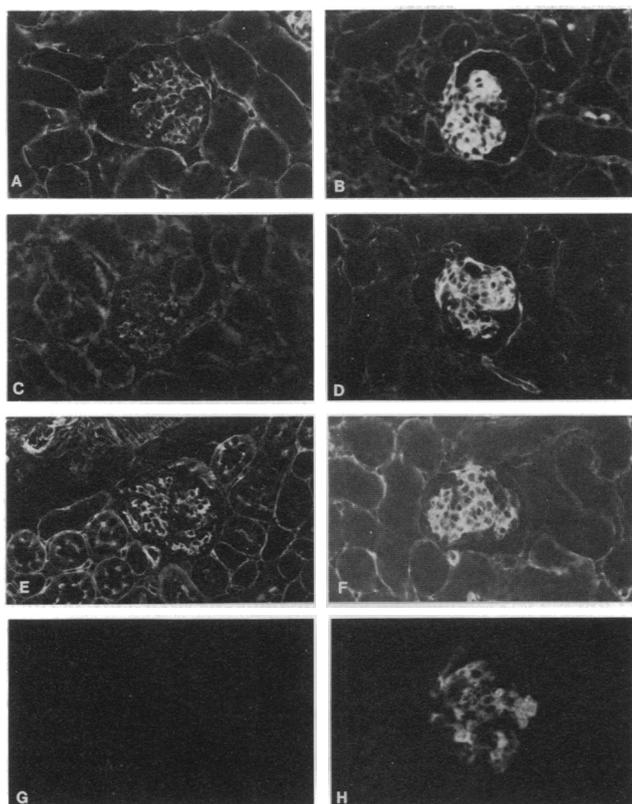


FIG. 4. Indirect immunofluorescent staining for extracellular matrix proteins. Renal tissue from transgenic mice aged 34 days exhibited increased staining of the mesangium for laminin (B), collagen type IV (D), and heparan sulfate proteoglycan (F) compared to the staining of the normal littermates for the same proteins (A, C, and E). Rev protein is present in sclerotic glomeruli in the kidney of a 53-day-old transgenic mouse (H), whereas an age-matched normal mouse kidney shows no staining (G). Photomicrographic exposures of normal and transgenic kidney sections were of the same duration. ( $\times 150$ )

likely responsible for the nephropathy reported here, as identical renal disease was observed in three lines, and each line was characterized by a unique restriction fragment length polymorphism. The absence of renal disease in five of the eight lines bearing the viral construct described here may be a consequence of insertion of the transgene in these instances into regions of the mouse genome that are transcriptionally silent in the kidney. Similarly, renal disease was not found in mice transgenic for the whole HIV-1 genome in work by Leonard *et al.* (23). This could have been the result of transgene insertion into a genomic site that is transcriptionally silent in kidney or, alternatively, the result of premature death in these mice, which all died by 25 days. Thus, the development of nephropathy in HIV-transgenic mice may require both the appropriate viral genes and tissue-specific gene expression.

In summary, the development of nephropathy in mice transgenic for HIV-1 genes implicates HIV-1 gene products in the pathogenesis of HIV-associated nephropathy. This small-animal model provides a unique opportunity to determine the particular viral gene products responsible for progressive glomerulosclerosis and to investigate strategies involving gene therapy to prevent this complication.

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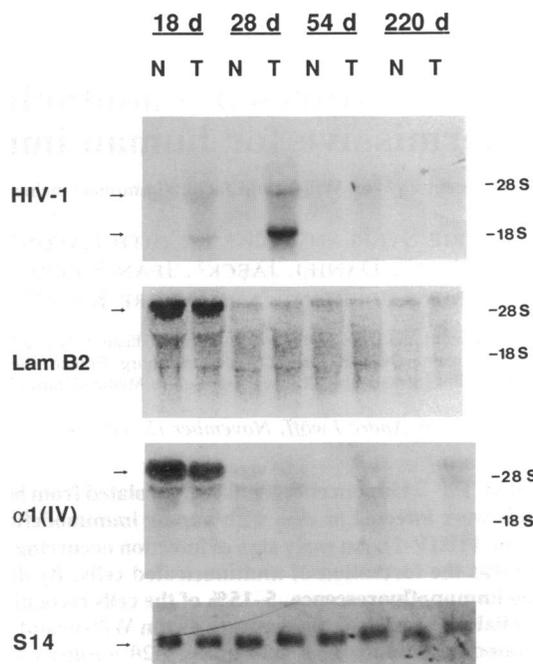


FIG. 5. Northern blot analysis of HIV-1 gene expression and extracellular matrix protein gene expression. Total renal RNA was hybridized with labeled cDNA probes encoding *nef*, mouse laminin B2 chain, mouse collagen  $\alpha 1(IV)$ , and human ribosomal protein S14. The 2-kb and 4-kb HIV-transgene mRNA species and the laminin, collagen, and S14 ribosomal mRNAs are indicated by arrows. The positions of the 28S and 18S rRNAs are also indicated. d, Days; N, normal; T, transgenic.

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