

The Age-Related Increase in Renal Clusterin mRNA Is Accelerated in Obese Zucker Rats

NICHOLAS J. LAPING,* BARBARA A. OLSON,* JONATHAN R. DAY,[‡]
BRIDGET M. BRICKSON,* LISA C. CONTINO,* BRIAN G. SHORT,[†]
SHUJATH M. ALI,* and DAVID P. BROOKS*

Departments of *Renal Pharmacology and [†]Morphologic Pathology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania; and [‡]Department of Biology, Mueller Laboratory, Pennsylvania State University, University Park, Pennsylvania.

Abstract. Clusterin is a multifunctional glycoprotein associated with development and tissue injury. Because renal function decreases with advancing age in the obese Zucker rat, clusterin mRNA expression was examined in the kidney of young adult Zucker rats and compared with age-related changes in renal clusterin mRNA expression in Fischer 344 (F344) rats. Renal clusterin mRNA levels in the obese Zucker rat were 2.5-fold higher by 3 mo of age and fourfold higher at 5 mo of age compared with the lean strain. In comparison, renal clusterin mRNA in 12-mo-old F344 rats was twofold higher than in 3-mo-old animals and was tenfold higher at 24 mo of age.

Clusterin mRNA was positively correlated with urinary protein excretion and negatively correlated with creatinine clearance in Zucker rats. Clusterin was increased in select nephrons of the obese Zucker rat kidney and in 24-mo-old F344 rat kidney as assessed by *in situ* hybridization. Increased expression of clusterin mRNA occurred mostly in the tubular epithelium of dilated, convoluted proximal tubules. These data indicate that renal clusterin mRNA levels increase as a function of age and that age-related increases in renal clusterin and the associated tubular abnormalities are accelerated in obese Zucker rats. (J Am Soc Nephrol 9: 38–45, 1998)

Clusterin is a multifunctional protein also known as sulfated glycoprotein-2 (1), testosterone repressed message-2 (2), apolipoprotein-J (3), serum protein 40,40 (4), gp 80 (5), and glycoprotein-III (6). Clusterin is a heterodimeric glycoprotein that is expressed in many tissues and is a component of HDL (3,7). It is expressed in a secreted form and a nonsecreted form with a nuclear localization signal (8). This protein appears to have other functions, which include apoptosis, neurodegeneration, inhibition of complement-mediated cell lysis, and kidney development (reviewed in references 9 through 11).

Clusterin mRNA levels are high in epithelial cells of many organs during development (11) and after injury or disease. For example, within the kidney, clusterin mRNA levels increase after ureteral obstruction (12) and after reduction in renal mass (13). High levels of clusterin are also expressed in the collecting duct cysts in polycystic kidney disease (14), and clusterin immunoreactivity is found in complement and immune deposits in glomerulonephritis (4,5). The relative expression of clusterin in diabetic nephropathy is not known.

Diabetic nephropathy is marked by persistent proteinuria and progressive loss of renal function without ischemia. The obese Zucker rat has mild glucose intolerance and peripheral

insulin resistance similar to type II diabetes (15). The obese Zucker rat is used as a model of hyperinsulinemic obesity, which results in kidney enlargement, proteinuria, focal segmental glomerulosclerosis, and loss of renal function (16,17). It is not known whether renal clusterin expression changes in the Zucker rat as a function of renal disease. In the present studies, therefore, we have examined clusterin mRNA in Zucker rat kidneys by Northern blot analysis and *in situ* hybridization. Because renal function decreases with increased age in the obese Zucker rat, we also examined clusterin mRNA levels in aging F344 rats, a nondiabetic, nonobese rat strain.

Materials and Methods

Animals

Male Zucker rats (3-, 4-, and 5-mo-old) of lean and obese strains and male Fischer 344 (F344) rats (3-, 12-, and 24-mo-old) were housed under controlled light, temperature, and humidity conditions. Animals were killed with 120 mg/kg pentobarbital, and kidneys were removed. Kidneys were collected and frozen on dry ice for RNA extraction or were snap-frozen in liquid nitrogen for *in situ* hybridization. Urine samples were collected for 24 h in metabolic cages from Zucker rats. Urine protein levels were determined by the sulfosalicylic acid method (18). Urine and plasma creatinine, urea nitrogen, potassium, and sodium levels were measured by Synchron Clinical System AS 8 (Beckman, Palo Alto, CA). Because the F344 rats were part of an ongoing aging study in which neuronal parameters were the primary end points, no renal functional data had been collected. All animals were treated in accordance with National Institutes of Health guidelines for the use of animals in research. All procedures were approved by the appropriate institutional animal care committee.

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Correspondence to Dr. Nicholas J. Laping, Department of Renal Pharmacology, SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406.

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Northern Hybridization

Total RNA was extracted from whole kidney by guanidinium thiocyanate denaturation and acidified phenol-chloroform extraction (19). Total RNA (10 μ g) was fractionated on 0.2 M formaldehyde-1% agarose gels and transferred to nylon membranes (Nylon-1, Life Technologies, Gaithersburg, MD) in $4\times$ SSC. Equivalent loading and transfer were verified by methylene blue staining and vacuolar H^+ -ATPase mRNA levels. Antisense [32 P]cRNA clusterin probe recognizes an mRNA at 2.0 kb. The clusterin clone was a generous gift from Dr. M. Griswold (Washington State University, Pullman, WA). The rat equivalent cDNA sequence for human and bovine vacuolar H^+ -ATPase was cloned from rat renal cortex mRNA by differential display PCR, using primers 5'-CTCACAGTCATCC-3' and 5'-AC-CACACATCTGA-3'. This clone is a 280-nucleotide insert cloned into pGEM 5zf vector (Promega, Madison, WI) and has 98% identity with bovine vacuolar H^+ -ATPase. Random-primed [32 P]cDNA were made and recognized a single 2.9-kb transcript. Hybridizations were performed with 10^6 cpm/ml in 50% formamide, 225 mM NaCl, 20 mM NaH_2PO_4 , 1.5 mM EDTA, 1% sodium dodecyl sulfate (SDS), 0.5% dry milk, 100 mg/ml yeast total RNA, and 300 mg/ml salmon DNA at 55°C for 15 h. Blots were washed with final stringency of $0.2\times$ SSC, 0.2% SDS at 72°C for the clusterin riboprobe and 42°C for the vacuolar ATPase DNA probe. Membranes were exposed to phosphorimaging plate, and bands were quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Statistical significance was determined by ANOVA (SuperANOVA software, Abacus Concepts, Berkeley, CA).

In Situ Hybridization

Thaw-mounted cryostat sections from lean and obese Zucker rats or 3- and 24-mo-old F344 rats (12 μ m) were fixed in 4% paraformaldehyde for 30 min. Sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, dehydrated in ethanol series, and incubated with 0.3 ng/ μ l per kb of 33 P-labeled clusterin cRNA probe in the hybridization solution (50% formamide, $4\times$ SSC, $5\times$ Denhardt's, 10% dextran sulfate, 1% SDS, and 250 μ g/ml transfer RNA) overnight at 50°C under baked glass coverslips. Coverslips were removed in $4\times$ SSC, and nonspecific hybridizations were reduced by incubating slides in 0.025 mg/ml RNase A (in 0.5 M NaCl and 0.05 M phosphate buffer) at 37°C for 30 min. The criterion wash was performed at 60°C for 30 min (50% formamide, 0.5 M NaCl, and 50 mM sodium phosphate, pH 7.4). Dehydrated slides were dipped in NTB-2 emulsion (Kodak) and exposed for 3 d. Developed slides were counterstained with hematoxylin and eosin.

Results

Clusterin mRNA levels were elevated in obese rats at 3, 4, and 5 mo of age compared with lean rats (Figure 1). Vacuolar H^+ -ATPase mRNA, however, did not change as a function of age in lean or obese Zucker rats (Figure 1). Clusterin mRNA levels were higher in the obese strain compared with the lean strain by 2.5-fold at 3 mo of age and by fourfold at 5 mo of age (Figure 2A). Clusterin mRNA levels were also increased in lean Zucker rats as a function of age: 1.7-fold higher at 4 mo and 2.5-fold higher at 5 mo (Figure 2A). Note that the age-related increase in clusterin mRNA levels was accelerated in the obese Zucker strain.

To determine whether parameters of renal function correlated with changes in clusterin expression, urinary protein excretion and creatinine clearance were examined in lean and

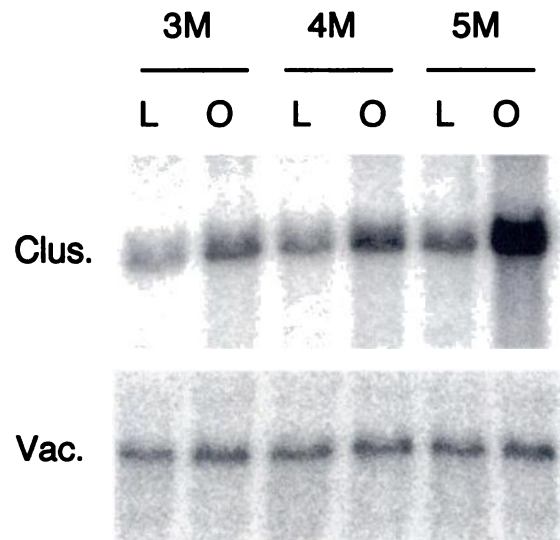


Figure 1. Northern blot of clusterin (Clus.) and vacuolar (Vac.) H^+ -ATPase mRNA in kidneys of lean (L) and obese (O) Zucker rat RNA (10 μ g of total RNA/lane) at 3, 4, and 5 mo of age demonstrates elevated clusterin mRNA levels in obese rats compared with lean rats at all three ages.

obese Zucker rats. Urinary protein excretion increased in obese rats over sixfold between 3- and 5-mo-old obese Zucker rats (Figure 2B). The increased protein excretion was positively correlated with increased clusterin mRNA levels ($r = 0.86$; Figure 3). At 5 mo of age, creatinine clearance was significantly lower in obese rats compared with lean animals (Table 1). In addition, sodium and potassium fractional excretions were significantly elevated in the obese Zucker rat, offering further evidence of a reduction in renal function (Table 1).

To evaluate whether the age-related changes in obese Zucker rats were also present in senescent "nondiabetic" rats, clusterin expression was examined in 3-, 12-, and 24-mo-old F344 rats. In this strain of rats, clusterin mRNA was elevated only twofold at 12 mo compared with 3-mo-old rats. However, at 24 mo of age, clusterin mRNA was tenfold higher than in 3-mo-old F344 rats (Figure 4).

Clusterin mRNA expression was detected in the renal cortex, medulla, and papilla of lean and obese Zucker rats by *in situ* hybridization. Focal, dilated renal cortical tubules had the most intense clusterin expression. Dilated tubules with clusterin expression were rare in lean 3- or 4-mo-old rats (Figure 5A), but were more common in 3-mo-old obese rats (Figure 5B). In 4-mo-old obese rats, there was a progression to cystic cortical tubules, and many of these cystic tubules had intense clusterin mRNA expression (Figure 5C). The histological changes in 5-mo-old animals occurred with higher frequency, but in all other respects were similar to those observed in 4-mo-old animals (not shown). Occasional sites of intense clusterin expression in kidneys of 3-mo-old obese rats were observed over the proximal tubule, particularly the initial portion of the proximal convoluted tubule segment near the glomerulus (S1) (Figure 5D). This finding, and the localization of clusterin-positive tubules to the cortex and outer stripe of the

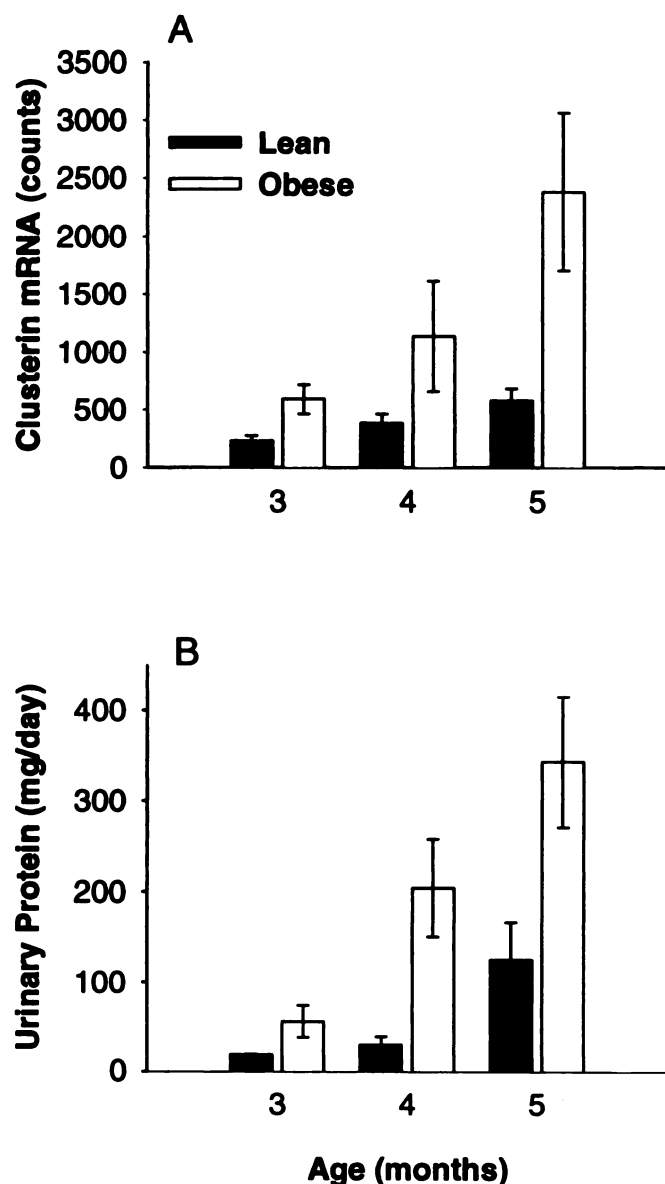


Figure 2. Clusterin mRNA and urinary protein excretion in lean and obese Zucker rats. (A) Bars represent clusterin mRNA levels (mean \pm SEM) in lean (black) and obese (white) Zucker rats at 3, 4, and 5 mo of age (effect of age $P < 0.01$ and effect of obesity $P < 0.005$, by ANOVA). (B) Bars represent urinary protein excretion (mean \pm SEM) in lean and obese Zucker rats at 3, 4, and 5 mo of age (effect of age $P < 0.01$ and effect of obesity $P < 0.005$, by ANOVA).

outer medulla in kidneys from 3-mo-old obese rats, suggests that the initial increases in renal clusterin expression occurred in the proximal tubule. Generally, glomerular clusterin expression was similar between lean and obese rats, but occasional glomeruli from clusterin-positive nephrons demonstrated increased expression in parietal epithelial cells of Bowman's capsule (Figure 5D) or near the macula densa (not shown). Increased clusterin mRNA levels in the parietal epithelial cells did not correlate with dilated Bowman's capsules.

Small, focal increases in clusterin mRNA expression were observed over distal tubules or collecting ducts in the inner and

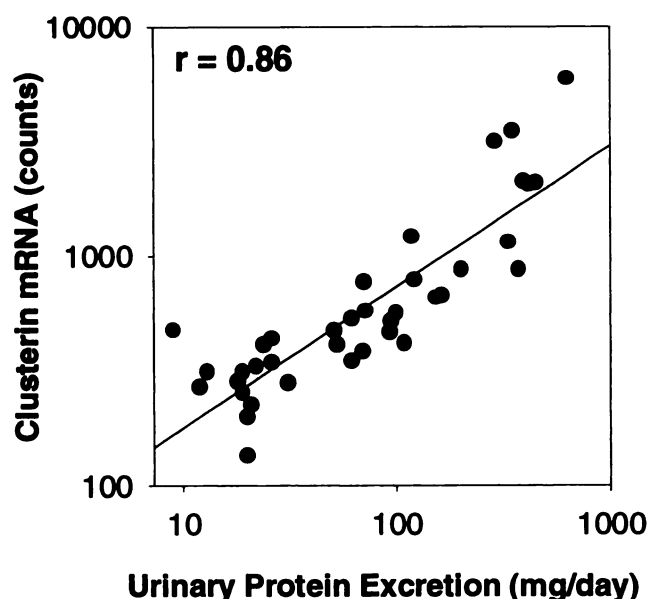


Figure 3. The progression of urinary protein excretion (mg/d) from individual Zucker rats as a function of renal clusterin mRNA levels. Animals from all groups (obesity and age) are represented. Urine protein excretion correlates with clusterin mRNA expression ($r = 0.861$, $P < 0.0001$).

Table 1. Renal function in 5-mo-old lean and obese Zucker rats^a

Parameter	Lean	Obese
Creatinine clearance (ml/min per 100 g)	0.50 \pm 0.04	0.27 \pm 0.04 ^b
Serum UN (mg%)	19.4 \pm 0.5	26.3 \pm 2.8 ^c
UN clearance (ml/min per 100 g)	0.35 \pm 0.03	0.23 \pm 0.03 ^c
FeNa (%)	0.5 \pm 0.02	1.0 \pm 0.25 ^c
FeK (%)	37 \pm 2	65 \pm 9 ^d

^a UN, urea nitrogen.

^b $P < 0.001$ by ANOVA.

^c $P < 0.05$.

^d $P < 0.01$.

outer stripe of the outer medulla from kidneys of 3- and 4-mo-old lean rats (Figure 6A). Larger, more intense increases in this region were observed in 4-mo-old obese rats (Figure 6B).

In the papilla, linear rays of slightly increased clusterin expression were observed in lean rats (Figure 6C), and more intense focal clusterin expression was observed in obese rats (Figure 6D). Collecting ducts were the most likely source of this increased expression. Intense clusterin expression was observed over the urothelium of the pelvis and calyx in lean and obese rats at 3 and 4 mo (not shown).

Similar changes in tubular clusterin expression were observed in the 24-mo-old F344 rats, including intense clusterin mRNA signal in select dilated proximal tubules in the renal cortex (Figure 7, C and D), and collecting ducts in the medulla (not shown). The clusterin signal colocalized to most epithelial

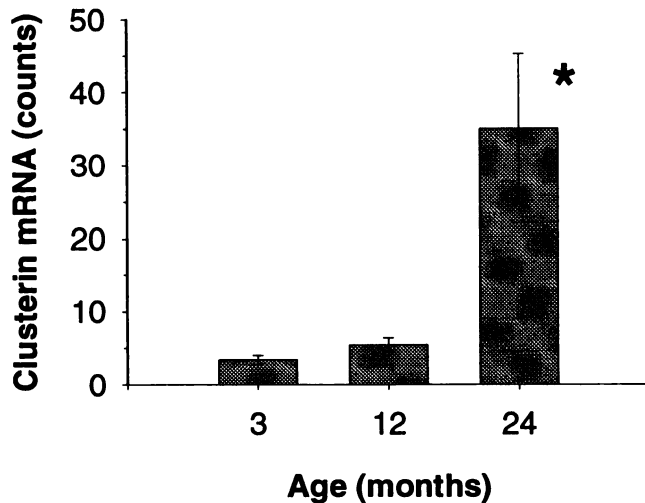


Figure 4. Northern blot of clusterin mRNA in 3-, 12-, and 24-mo-old Fischer rat (F344) kidneys. Bars represent clusterin mRNA levels (mean \pm SEM). *, main effect of age, $P < 0.01$ by ANOVA.

cells lining the distended tubules. As seen in Zucker rats, some 24-mo-old F344 rats had greater than 80% distended and degenerated tubules, whereas only 5 to 10% of those tubules showed increased clusterin mRNA, indicating that a select class of distended tubules expresses high levels of clusterin mRNA. In 24-mo-old F344 rats, increased clusterin mRNA was detected in parietal epithelial cells of mostly nondistended glomeruli. However, in those aged animals with severe pathology in which all Bowman's capsules were distended, no clusterin mRNA was detected in glomeruli (not shown). Diffuse clusterin expression was seen in 3-mo-old F344 rats, with occasional glomeruli showing increased signal (Figure 7, A and B).

Discussion

This study demonstrates that clusterin mRNA was increased in kidneys of obese Zucker rats when compared with lean Zucker rats as early as 3 mo of age. There was an age-related progressive increase in renal clusterin mRNA in lean Zucker rats and F344 rats that was accelerated in the obese animals. Clusterin mRNA appears to be a good indicator of decreased renal function, because clusterin mRNA levels were positively correlated with urinary protein excretion and negatively correlated with creatinine clearance, and were associated with tubular degeneration.

In the present study, clusterin mRNA was elevated in dilated cystic nephrons of the obese Zucker rat and in the aged F344 rat kidney, and occurred mostly in the epithelium of dilated cortical tubules. Not all dilated tubules, however, had high levels of clusterin mRNA, which suggests that clusterin may be expressed at a particular stage during the tubular remodeling process. This hypothesis is supported by strong developmental expression of clusterin in epithelial cells of the kidney during embryogenesis (11). Thus, clusterin expression could be a compensatory response during tubular degeneration.

The pattern of high clusterin mRNA levels in select tubules of the obese Zucker rat and aged F344 rat kidney reported in

this study was also similar to clusterin expression observed in human kidneys after ischemia, in which clusterin is colocalized with complement C9 in brush-border-positive proximal tubules (20). This observation is consistent with the ability of clusterin to block complement-mediated cell lysis by binding complement C9 (21,22). Because clusterin is colocalized with complement proteins in patients with kidney disease (20,23), it is possible that the clusterin-positive atrophic nephrons observed in the obese Zucker rat and aged F344 rat may have been under complement attack. Additional colocalization studies are required to address this hypothesis.

Although clusterin is elevated after injury in many different organ systems, its function during tissue repair processes is unknown. A protective role for clusterin has been suggested by Rosenberg and Silkens (10). They have demonstrated that clusterin causes the aggregation of renal epithelial cells in culture (24) and concluded that clusterin might mediate cell-cell and cell-substratum interactions, preventing leakage of fluids and cells at tissue-fluid interfaces. Additional examples of clusterin's potential protective role include studies in which clusterin depletion leads to enhanced immune glomerular injury in isolated perfused kidneys (25). Clusterin also appears to protect the androgen-sensitive prostate cancer cell line from tumor necrosis factor- α -induced cell death (26). Thus, blocking the expression of clusterin with antisense oligonucleotides enhances cell death, whereas transfection of androgen-sensitive prostate cancer cells with a clusterin-containing expression vector prevents tumor necrosis factor- α -induced cell death (26). Because clusterin blocks complement-mediated cell lysis (21,22), the increased expression of clusterin is a potentially protective effect in the impaired obese or aged rat kidney.

The obese Zucker rat is hyperlipidemic, which likely contributes to the loss of renal function over time. The development of proteinuria and focal glomerulosclerosis in the obese Zucker rat can be reduced by decreasing caloric intake or by inhibiting cholesterol synthesis (27,28). Whether similar treatments of lipid reduction also alter the age-related changes in renal clusterin expression of the F344 rat remains to be determined. It has been shown, however, that caloric restriction, and not dietary protein restriction, extends lifespan and retards other age-related changes, including renal function in rodents (29–32). The mechanism by which hyperlipidemia causes renal damage and subsequent clusterin mRNA elevation is unclear.

In summary, clusterin mRNA is higher in the obese Zucker rat and aged F344 rat kidney. Clusterin mRNA increases as a function of age in both lean and obese rats and is positively correlated with tubular abnormality, proteinuria, and reduced renal function. This study suggests that progressive loss of renal function due to hyperlipidemia is associated with a progressive increase in the number of dedifferentiated nephrons expressing clusterin mRNA. Because it has been shown that clusterin is also highly expressed in renal epithelial cells during kidney development (11), it is likely that intense remodeling was occurring in those select nephrons identified by clusterin in the compromised kidney. Although much evidence in other organ and model systems describes a protective role for clus-

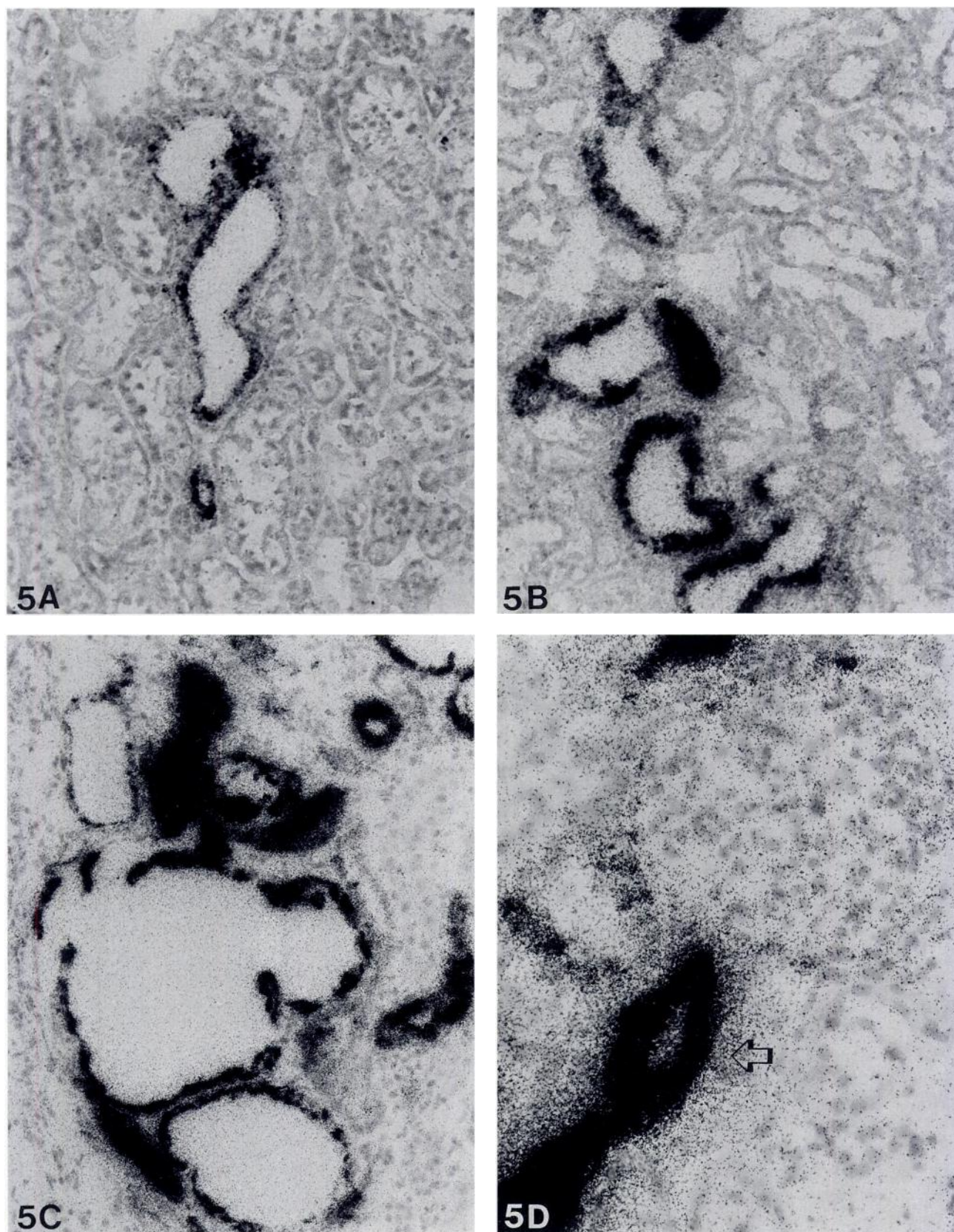


Figure 5. *In situ* hybridization photomicrographs of clusterin mRNA in kidney cortex from lean and obese Zucker rats. (A) Three-month-old lean rat: Rare, dilated tubule demonstrates increased signal. (B) Three-month-old obese rat: Increased number of dilated tubules from one nephron with increased clusterin expression. (C) Four-month-old obese rat: Many cystic, dilated tubules with increased clusterin expression. (D) Intense clusterin expression over S1 segment of proximal tubule (arrow) and slight increase in expression in glomerulus and parietal cells of Bowman's capsule of 3-mo-old obese rat. Magnification: $\times 200$ in A through C; $\times 400$ in D.

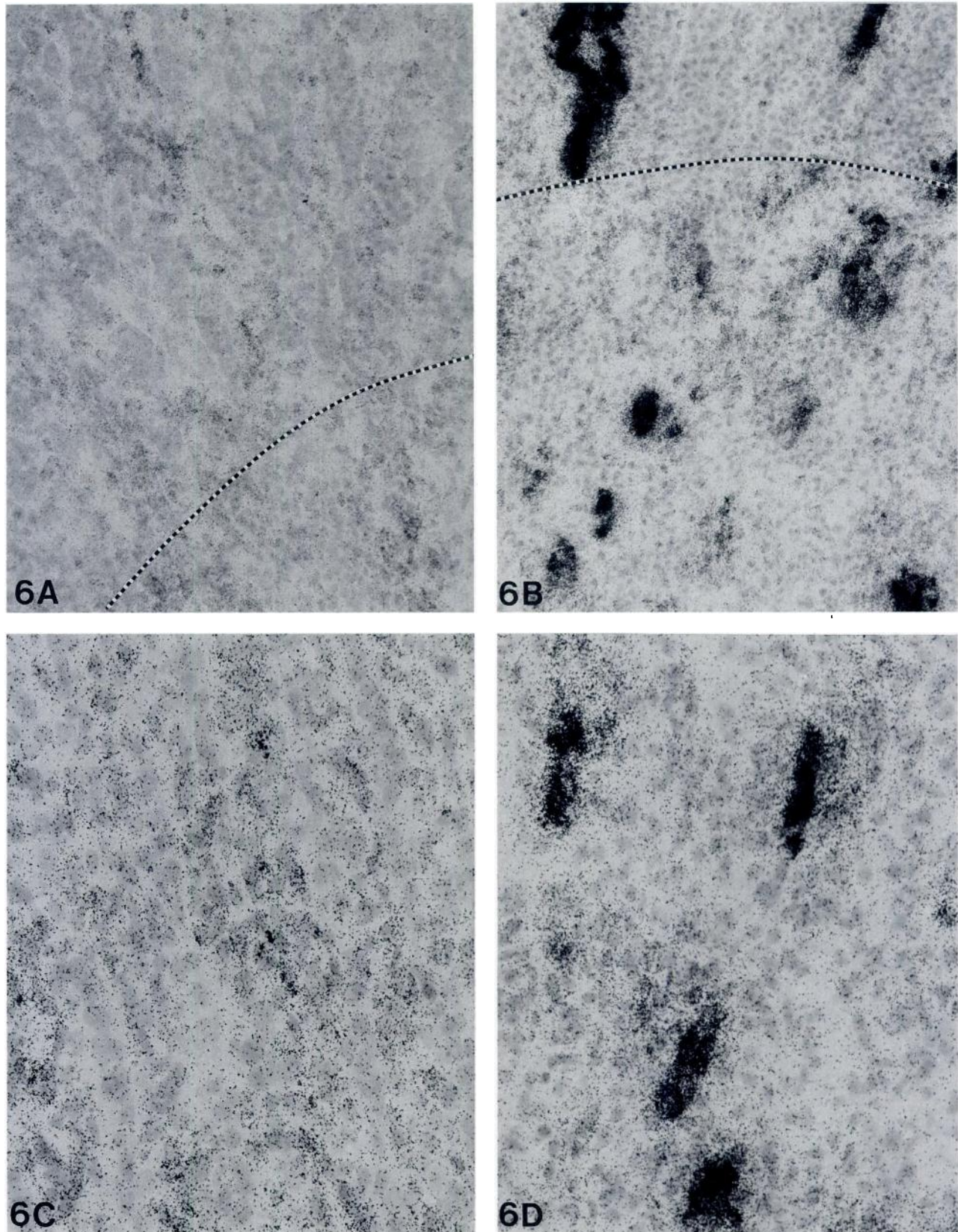


Figure 6. *In situ* hybridization photomicrographs of clusterin mRNA in tissue sections of kidney medulla (A and B) and papilla (C and D) from lean and obese Zucker rats. (A) Medulla of 3-mo-old lean rat: Slightly increased signal over distal tubules or collecting ducts above or below junction of inner and outer stripe of outer medulla (dotted line). (B) Medulla of 4-mo-old obese rat: Focal, intense signal over proximal tubule or collecting duct in outer stripe of outer medulla (above dotted line) and distal tubule or collecting duct in inner stripe of outer medulla (below dotted line). (C) Papilla of 3-mo-old lean rat: Linear rays of slightly increased clusterin expression. (D) Papilla of 4-mo-old obese rat: Focal, intense expression of clusterin in collecting duct. Magnification: $\times 200$ in A and B; $\times 400$ in C and D.

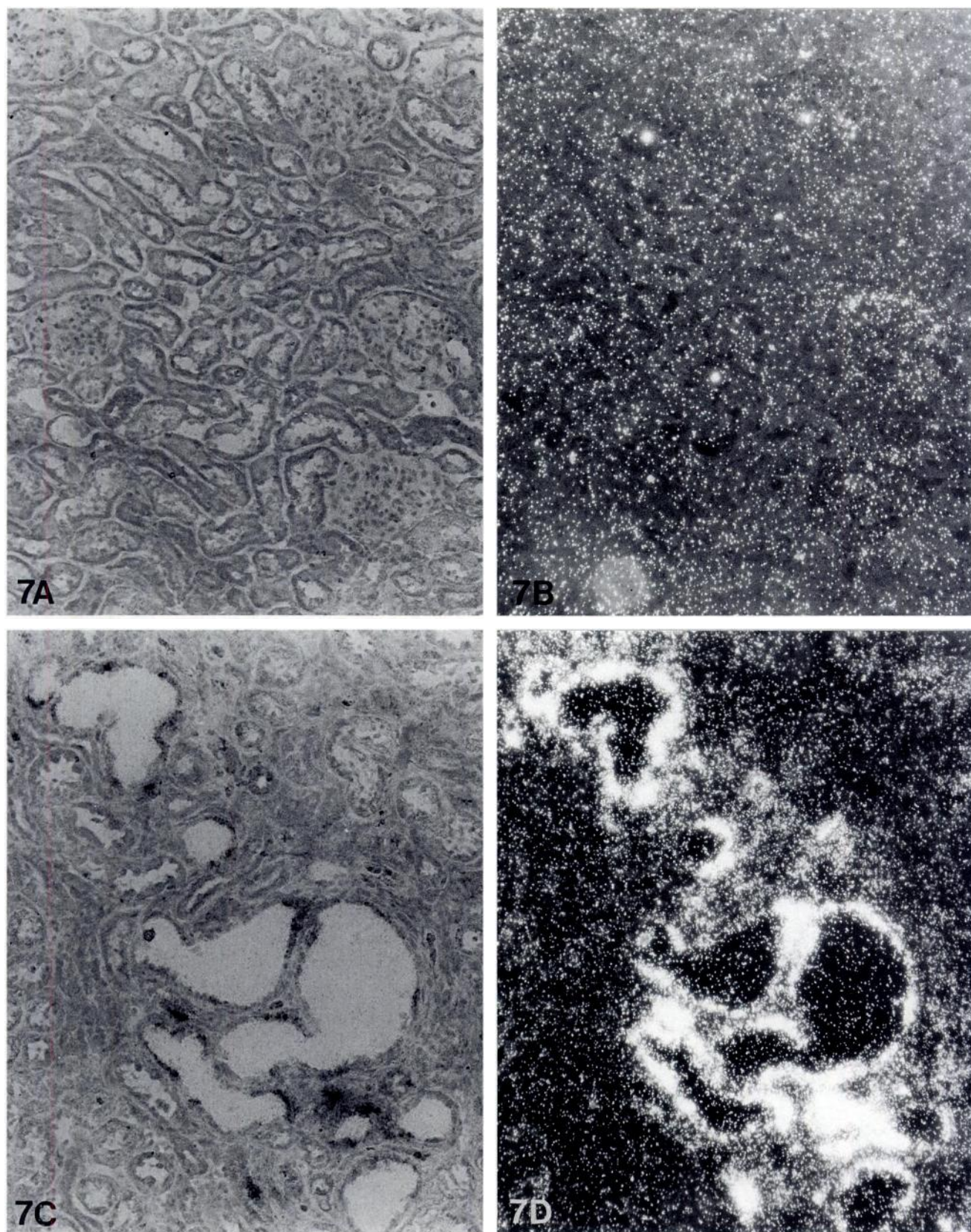


Figure 7. *In situ* hybridization photomicrographs of clusterin mRNA in tissue sections of kidney cortex from 3-mo-old (A and B) and 24-mo-old (C and D) F344 rats. A and C, bright field; B and D, dark field. (A and B) Diffuse clusterin signal in 3-mo-old rat kidneys with occasional increased signal in glomeruli. (C and D) Focal, intense signal over dilated proximal tubule in 24-mo-old renal cortex. Clusterin is localized over the tubular epithelium. Magnification, $\times 100$.

terin, its function in renal tubular degeneration remains to be determined.

Acknowledgments

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