

Expression of an Epidermal Keratin Protein in Liver of Transgenic Mice Causes Structural and Functional Abnormalities

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Abstract. To examine the role of keratin intermediate filament proteins in cell structure and function, transgenic mice were isolated that express a modified form of the human K14 keratin protein in liver hepatocytes. A modified K14 cDNA (K14.P) sequence was linked downstream of the mouse transthyretin (TTR) gene promoter and enhancer elements to achieve targeted expression in hepatocytes. Hepatocytes expressing high levels of the transgene were found to have abnormal keratin filament networks as detected by indirect immunofluorescence using an antibody specific for the transgene product. Light and electron microscopic level histological analysis of isolated liver tissue showed in many cases degenerative changes that in-

cluded inflammatory infiltration, ballooning degeneration, an increase in fat containing vacuoles, and glycogen accumulation. These changes were most evident in older mice over four months of age. No indication of typical Mallory body structures were identified at either the light or electron microscopic level. To evaluate secretory function in transgenic livers, bile acid secretion rates were measured in isolated perfused liver and found to be approximately twofold lower than aged-matched controls. These findings indicate that expression of an abnormal keratin in liver epithelial cells in the *in vivo* setting can alter the structure and function of a tissue and suggest a role of the keratin network in cellular secretion.

INTERMEDIATE filament (IF)¹ proteins comprise a multigene family of five major types that are expressed in a tissue-specific manner (for review see Albers and Fuchs, 1992; Fuchs and Weber, 1994). Keratin proteins are the largest and most complex IF group and are expressed as specific pairs during epithelial cell development and differentiation (Franke et al., 1982; Fuchs et al., 1987). Keratin polypeptides assemble into 10–14-nm diam filaments that form an extensive cytoplasmic network with associations at the nuclear and cytoplasmic membranes.

Keratins are classified as type I or type II proteins based upon their charge, immunological relatedness, and sequence similarity (Fuchs et al., 1981; Moll et al., 1982). Type I keratins (K9–K20) are generally smaller in molecular mass and more acidic (molecular mass 40–56.5 kD; pK_i 4–6) than type II keratins (K1–K8), which are larger and neutral basic (molecular mass 53–67 kD; pK_i 6–8). Type I and type II subunits are expressed as pairs that copolymerize into heteropolymer structures (Sun et al., 1984). Pairing specificity is thought to create networks tailored to the function and phenotypic features of an epithelium (Eichner et al., 1986).

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1. *Abbreviations used in this paper:* EH, epidermolytic hyperkeratosis; IF, intermediate filament; SP, substance P; SRm, secretory rate; TC, taurocholate; TTR, transthyretin.

For example, basal keratinocytes of the stratifying epithelia of the epidermis express the type I K14 and type II K5 proteins. These keratins assemble into high affinity filament complexes (Coulombe and Fuchs, 1990; Quinlan et al., 1985) that form stable, resilient networks that impart strength to the epidermis and facilitate its role as a protective organ. In contrast, simple epithelial cells contain keratin filaments composed of keratins K18 (type I) and K8 (type II) that differ from skin keratins by having greater flexibility and solubility (Chou et al., 1993). These physical properties are thought to be more adaptive to simple epithelium, i.e., keratin filaments may serve less of a protective role and more as cytoplasmic organizers.

Numerous studies and experimental approaches have been used to understand the role of keratin protein pairing specificity and function. *In vitro* assembly studies have demonstrated that many combinations of type I and type II subunits polymerize to form morphologically similar filaments (Hatzfeld and Franke, 1985). Studies using cultured cells transfected with various cloned keratin genes have shown that expression of ectopic keratins in epithelial cells does not overtly perturb cell morphology and survival (Albers and Fuchs, 1987, 1989; Domenjoud et al., 1988; Kulesh and Oshima, 1988; Lersch et al., 1989; Blessing et al., 1989), suggesting that alteration of network composition was not detrimental. However, studies using retroviral promoter driven expression of various type I and type II keratin pairs in 3T3 fibroblasts, cells that normally contain vimentin net-

works, have shown that some keratin pairs form more extended filaments than others (Lu and Lane, 1990). For example, K8 and K18 proteins assembled into an extended filament network whereas K8 and K14 proteins formed an irregular, fragmented network.

To examine the requirements of keratin filament composition in tissue function, we have isolated lines of transgenic mice that express a modified epidermal K14 keratin in the simple epithelial hepatocytes of the liver. A derivative of the human K14 epidermal keratin cDNA, K14.P (Albers and Fuchs, 1987) was expressed in liver using promoter and enhancer sequences from the gene encoding the liver expressed serum protein, transthyretin (TTR) (Costa et al., 1990). We targeted K14.P expression to liver because it is a large organ that is well characterized structurally and biochemically and because in several disorders of liver, the hepatocyte IF network becomes disorganized into aggregate structures known as Mallory bodies. Mallory bodies are frequently found in a perinuclear array, contain cross-linked keratin proteins not normally expressed in liver (Denk et al., 1986; Zatloukal et al., 1992), and are typically found in liver diseases in which hepatocyte injury is a characteristic feature, e.g., alcoholic liver disease, Wilson's disease, chronic biliary diseases, and hepatocellular carcinoma (Denk et al., 1986; Cotran et al., 1989). The diversity of hepatic disorders exhibiting keratin abnormalities suggest that the integrity and composition of the keratin network has an intrinsic role in hepatocyte structure and function. To test this hypothesis we examined the histology and function of liver tissue from TTR-K14.P expressing transgenic mice. Results show that expression of the K14.P protein in liver could perturb the endogenous keratin filament network organization and produce various histopathologic damage. To determine whether these morphological abnormalities affected liver function, bile secretory function of TTR-K14.P transgenics was assayed. Transgenic mice from two separate lines had reduced bile flow rates compared to controls, suggesting that perturbation of the keratin intermediate filament network disrupts the normal secretory process in hepatocytes.

Materials and Methods

Construction and Purification of the TTR-K14.P Transgene

A HindIII fragment containing the K14.P coding and 3' untranslated sequences was cloned into the HindIII site of the plasmid pTTR-EP (a gift from R. Costa, University of Illinois, Chicago). Plasmid pTTR-EP contains the promoter and enhancer regions of the mouse transthyretin gene cloned into the plasmid pGem3Z (Promega, Madison, WI). To enhance expression of the K14.P cDNA transgene, ~2 kbp of the human growth hormone gene containing intron, exon, and a polyadenylation signal sequences (Palmiter et al., 1991), were ligated at the 3' end of the K14.P cDNA to generate plasmid pG3TT-K14.P (Fig. 1). The EcoRI TTR-K14.PhGH containing fragment was purified and used for generation of transgenic mice. Before injection the EcoRI fragment was electrophoresed through an 0.8% Sea plaque gel (FMC Bioproducts, Rockland, ME), extracted from the gel using GeneClean (Bio 101), phenol/chloroform extracted, ethanol precipitated and run through a NACS column (Bethesda Research Laboratories, Gaithersburg, MD). DNA was ethanol precipitated and resuspended in PBS at a concentration of 5 µg/ml as determined using a fluorometer (Pharmacia LKB Biotechnology, Piscataway, NJ). Purified DNA was microinjected into single cell mouse embryos derived from B6xC3H F1 mice. Embryos were implanted into the oviduct of pseudopregnant females using standard surgical techniques (Hogan et al., 1986). Offspring were identified as transgenic by Southern hybridization of digested DNA isolated from tail (Southern,

1975). Progeny of founder mice were screened for the transgene using either slot blot hybridization and/or polymerase chain reaction analysis of DNA isolated from ear punches. Mice were housed under sterile conditions in microisolator cages and siblings of founders and facility sentinel animals were routinely screened for the presence of mouse hepatitis virus, pneumonia virus, sendai virus, and reo virus. All tested negative.

Gene Transfection

HepG2 hepatoma cells were cultured on glass chamber slides (Nunc) in a 3:1 mixture of DME:F12 media supplemented with 10% bovine calf serum (Hyclone Labs., Logan, UT). TTR-K14.P plasmid DNA was transfected into cells overnight using calcium phosphate precipitation (Graham and Van der Eb, 1973). The precipitate was removed and cells were washed twice with PBS after which culture media was added. At 48 h posttransfection cells were fixed and processed for immunolabeling.

Immunofluorescent Labeling Analysis

Cultured Cells. Transfected HepG2 cells were double immunolabeled using 1:300 dilution of a rabbit polyclonal antibody against the neuropeptide substance P (Wako Bioproducts, Richmond, VA) to detect the K14.P protein and a rat monoclonal antibody (Troma 2, undiluted) against K8 (Boller and Kemler, 1983) to detect the endogenous network. Primary antibody labeling was visualized using fluorescein-conjugated goat anti-rabbit IgG (Cappel, Malvern, PA) and Texas red-conjugated goat anti-rat IgG (mouse absorbed; Sera Labs., Crawley Down, Sussex, England) secondary antibodies. Labeled cells were detected using a Zeiss Axioscop microscope equipped with fluorescent optics.

Liver Tissue. Immunofluorescence analysis was performed on liver tissue that was frozen on dry ice and stored at -80°C until sectioned. Tissue sections 6-10-µm thick were cut using a cryostat, mounted on Glass Plus slides, and stored at -80°C. Slides were air dried for 15 min, fixed in -20°C acetone for 10 min, air dried, and placed in PBS. Immunolabeling to detect keratin filaments used antibodies to substance P, tromba 2, or a monospecific rabbit antibody made to K18 protein (Stellmach and Fuchs, 1989). Desmosome complexes were labeled using a rabbit antidesmoplakin I/II antibody (Pasdar and Nelson, 1988). Sections were incubated in primary antibodies 1-2 h at room temperature, washed three times in PBS (10 min each), and incubated with an appropriate secondary antibody fluorophore conjugate. Secondary antibodies were applied for 25 min at room temperature followed by washing with PBS. Double-label immunofluorescence analysis was performed by sequential incubation of sections with primary antibodies followed by appropriate secondary antibodies.

Light and Electron Microscopic Analysis

Mice were anesthetized by injection of avertin anesthesia (i.p.), killed by cervical dislocation, and liver tissues removed. For light level analysis, tissue was placed in either 4% paraformaldehyde or Histochoice fixative (Amresco Inc., Solon, OH). Tissue was fixed overnight, dehydrated through alcohols, embedded in paraffin wax, sectioned (8-10 µm), and stained with hematoxylin and eosin. To examine glycogen content fixed liver sections were stained using the periodic acid-Schiff procedure. To detect lipid content fresh frozen liver sections were fixed for 10 min at room temperature in 4% paraformaldehyde in PBS, washed, and stained in freshly filtered oil-red O in 70% isopropanol for 10 min. For electron microscopy, tissue was immersion fixed for 2 h at room temperature in 3% glutaraldehyde made in 0.1 M Sorensen's phosphate buffer (pH 7.3), processed for epon embedding and examined on a JEOL 100 electron microscope.

Northern Analysis of Transgene Expression

Total RNA was extracted from heart, brain, kidney, and liver tissues using Trizol solution (BRL). Tissues were disaggregated in Trizol using a polytron and RNA extracted and precipitated according to the manufacturers protocol. 20 µg of total RNA was electrophoresed through a 1.2% agarose formaldehyde denaturing gel, transferred overnight in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7) to Nytran membrane, baked 2 h at 80°C, and then hybridized at 68°C to a [³²P]CTP-labeled riboprobe made to the full-length sequence of the K14.P cDNA.

Keratin Protein Isolation and Immunoblot Analysis

Keratin proteins were isolated from liver essentially using the procedure of Achtstaetter et al. (1986). Briefly, up to 1 g of tissue was homogenized in

10 ml of homogenization buffer (96 mM NaCl, 8 mM KH₂PO₄, 5.6 mM Na₂PO₄·2H₂O, 1.5 mM KCl, 10 mM EDTA, 0.1 mM DTT, pH 6.8) using a polytron. Three volumes of very high salt buffer (2 M KCl, 200 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM βME, 0.5% Triton X-100, 0.3 mg/ml PMSF) were added, the suspension was stirred for 30 min at 4°C, and insoluble material pelleted by centrifugation at 10,000 g for 20 min. Two additional washes in high salt buffer were performed and the pellet was resuspended in 6 M urea, 50 mM Tris, pH 7.4, and 10 mM βME. Protein concentration was measured using a Bradford protein assay (Biorad Labs., Hercules, CA).

Immunoblot analysis was performed on protein samples separated on a 10% polyacrylamide gel. Proteins were electrophoretically transferred to a nitrocellulose membrane and processed for immunolabeling (Towbin et al., 1979). The membrane was exposed to the substance P antibody (1:300), washed, and incubated with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Antibody binding was detected by incubation with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate and color development reagent according to the manufacturer's instruction (Biorad Labs.).

Bile Flow Measures in Isolated Perfused Liver

Mice were anesthetized with 1 g/kg urethane i.p. and the liver perfused via the portal vein as described in Liu et al. (1992) with Krebs-Henseleit buffer (118.5 mM NaCl, 24.9 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.19 mM MgSO₄, 4.74 mM KCl, and 1.27 CaCl₂, pH 7.4) at a flow rate of 4 ml/min/g liver in a single pass perfusion system. The bile duct was cannulated with PE-10 tubing. Perfusate was oxygenated with 95% O₂/5% CO₂ and the liver maintained at 36°C. Bile was collected every 10 min throughout the experiment (70 min) and the volume determined gravimetrically assuming a density of 1.0. [³H]Taurocholate (TC; 8 nCi/μmol) was infused into the cannula leading to the portal vein at increasing concentrations for 10 min each. Inflow concentrations of TC were 56 ± 1.7, 108 ± 1.6, and 237 ± 13 nmol/ml. Aliquots of bile (10 μl) and perfusate (inflow and outflow; 200 μl each) were mixed with 5 ml Aquasol (New England Nuclear, Boston, MA) and assayed for radioactivity in a 1500 series Tricarb Liquid Scintillation Analyzer. The bile acid secretory rate (BASR) was calculated based on the formula BASR = BF × C, where BF is bile flow and C is bile acid concentration in the bile.

Results

Isolation of TTR-K14.P Transgenic Mice

To target expression of the K14.P keratin to liver hepatocytes the cDNA was placed downstream of a 400-bp sequence containing proximal promoter and enhancer elements of the mouse transthyretin (TTR) gene (Fig. 1 a). These regulatory sequences have been shown to be transcriptionally active in liver and have been used to direct transcription of a transthyretin minigene (Yan et al., 1990) and the creatine kinase gene (Koretsky et al., 1990) in transgenic mice.

To detect cellular expression of the transgene, a K14 cDNA fragment containing the carboxy tail of the neuropeptide substance P (SP) added at the 3' end was used (Albers and Fuchs, 1987). The P tag provided a specific immunologic epitope that allowed detection of transgene expression in liver hepatocytes. Recent studies in rat have found that K14 keratin is made by non-parenchymal liver epithelial cells (LECs) located in the biliary epithelium and Glisson's capsule (Blouin et al., 1992; Marceau et al., 1992). Thus the SP epitope provided a means to discriminate between cells expressing mouse K14 and cells expressing the K14.P transgene product.

Before injection of the TTR-K14.P DNA into mouse embryos, we tested whether the K14.P protein would assemble properly with the endogenous hepatocyte filament network by transfecting the pG3TTR-K14.P plasmid into the human hepatoma cell line HepG2 (Fig. 1 b). At 48 h, transfected cells were fixed and double labeled with the rabbit substance P antibody to detect the K14.P protein, and the rat anti-K8

antibody to detect the endogenous network (Fig. 1 b). Anti-SP labeling colocalized with the anti-K8-labeled endogenous network with no detectable perturbation in filament organization.

TTR-K14.P Transgene Expression in Liver Hepatocytes

Seven TTR-K14.P founder mice were bred to produce progeny for use in biochemical and histological analysis. No external physical differences were observed in the transgenic lines and thus far, all appear to have a normal lifespan. Copy number was estimated by densitometric measure of autoradiograms from slot blots on which the isolated transgene EcoR1 insert was loaded as a standard and ranged from one (line 454-1) to twelve copies (highest; 397-7). Five lines (397-7, 482-2, 484-4, 454-1, 454-2) were obtained that had detectable levels of transgene expression by Northern (not shown) and Western (Fig. 1 c) hybridization analysis. To detect transgene expression, intermediate filament proteins were purified from liver tissue using high salt extraction (Achtstaetter et al., 1986). The transgene product was not visible on Coomassie stained gels, though it was detectable using Western analysis (Fig. 1 c). The K14.P protein was detected using the substance P antibody followed by an alkaline phosphatase-conjugated anti-rabbit antibody. Binding was visualized by the appearance of alkaline phosphatase catalyzed substrate reaction product at the predicted size (~50 kD) of the K14.P protein. A fainter band was also detected above K14.P and may represent a product made using an upstream ATG added during plasmid construction that would add an additional 28 amino acids to the amino terminus. If this is the case, addition of these residues could affect filament assembly, though no indication of this was given by the morphology of the filament networks of hepatocytes transfected with the pG3TTRK14.P plasmid (Fig. 1 b).

The 397-7 and 482-4 line had the highest level of expression and were bred to produce mice homozygous for the transgene as judged by band intensities on slot blot and Southern hybridizations. Though these lines did exhibit the best level of expression, it was still quite low when compared to endogenous keratin levels; transgene expression was estimated to be less than 1% of the total extractable keratin protein by comparing band intensities densitometrically to known amounts of standard K14.P proteins purified from overexpressing bacteria (data not shown). The reason for this relatively low level of expression is not known.

The endogenous transthyretin gene is also normally expressed in the choroid plexus epithelium of the brain. It was of interest therefore to examine the tissue specificity of the TTR promoter and enhancer elements used in making the TTRK14.P transgenics. Total RNA was isolated from heart, brain, kidney, and liver tissues of a control and a 397-7 homozygous mouse and examined by Northern analysis using a riboprobe made to the full-length K14.P cDNA sequence (Fig. 1 d). For this line and the 482-4 line (not shown), transgene expression was only detected in the liver.

Incorporation of K14.P Causes Alteration of the Hepatocyte Keratin Network

To determine whether the K14.P keratin incorporated into the in vivo hepatocyte network, indirect immunofluorescence was used to label IF networks in transgenic and control

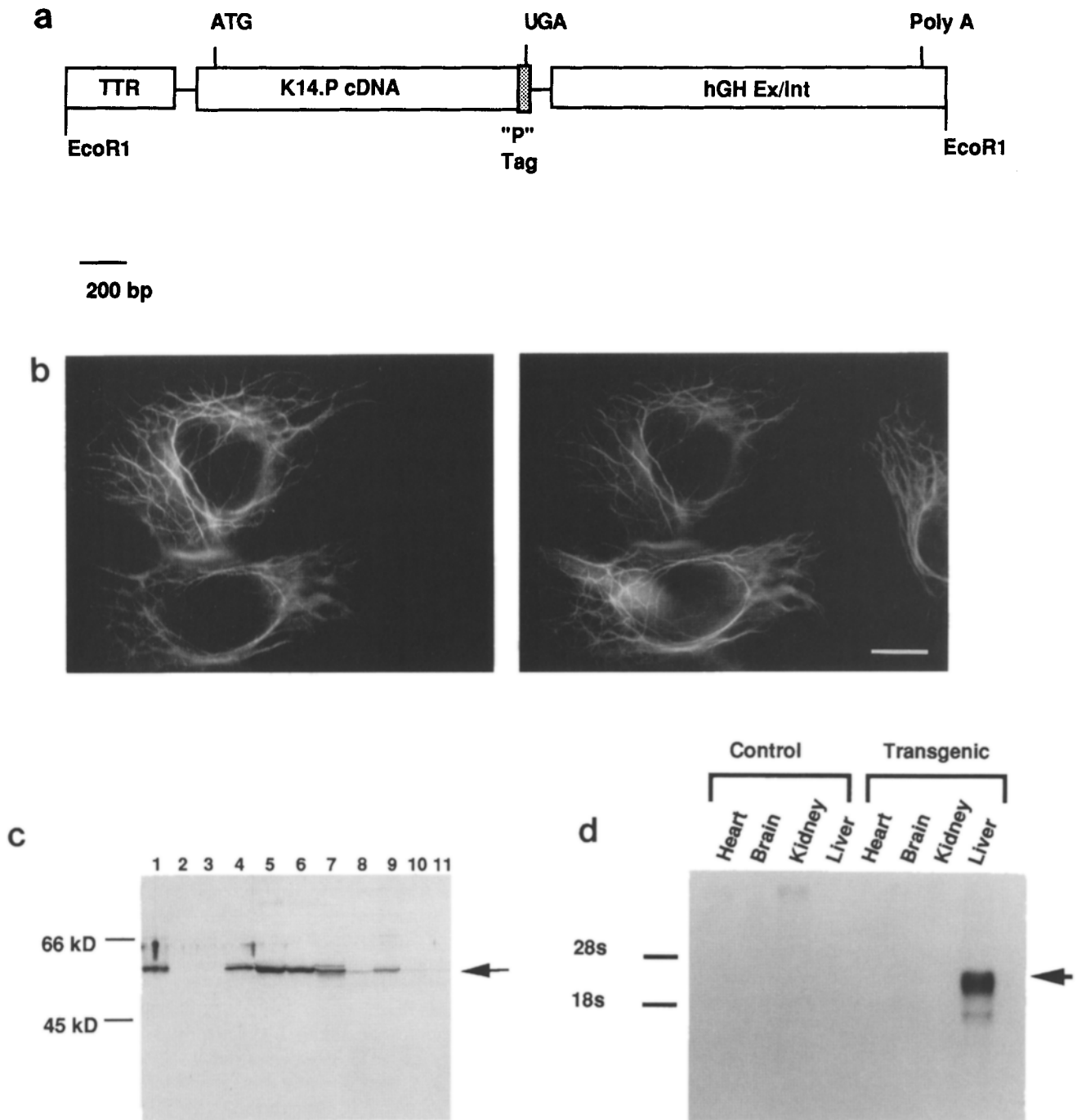


Figure 1. Genetic map and expression of the TTR-K14.P transgene. (a) The K14.P cDNA was targeted to mouse liver hepatocytes using elements of the transthyretin (TTR) gene promoter and enhancer. The P tag sequence encodes an antigenic portion of the neuropeptide substance P and serves as a specific marker of the transgene (Albers and Fuchs, 1987). The 3' sequences are from the human growth hormone gene (hGH; sequence from +2 to the poly A signal) which provide an intron/exon splicing segment that serves to upregulate expression and a polyadenylation signal. (b) To test expression of the plasmid and incorporation of the transgene protein into the endogenous network, pG3TTR-K14.P was transfected into HepG2 hepatoma cells. K14.P expression was visualized using double-label immunocytochemistry. Left panel shows cells labeled with the anti-SP antibody and an FITC-conjugated goat anti-rabbit secondary. Right panel is the same field labeled with the troma 2 rat monoclonal and a Texas red-conjugated goat anti-mouse secondary. Labeling was visualized using a Zeiss Axioskop microscope equipped with fluorescent optics. (c) Western blot to detect K14.P protein in transgenic livers using the substance P antibody. (Lane 1) K14.P marker protein (~50 kD) isolated from cells transfected with K14.P; (lane 2) no sample loaded; (lane 3) nontransgenic control, 20 μ g; (lane 4) 397-7F1 transgenic, 20 μ g; (lane 5) 397-7F2 transgenic, 20 μ g; (lane 6) 397-7F2 transgenic, 20 μ g; (lane 7) 397-7F2 transgenic, 20 μ g; (lane 8) 482-2F1 transgenic, 30 μ g; (lane 9) 482-4F1 transgenic, 30 μ g; (lane 10) 454-1F1 transgenic, 30 μ g; (lane 11) 454-2F1 transgenic, 30 μ g. Samples in lanes 5 and 6 were from homozygotes. (d) Northern blot shows tissue specificity of the TTR promoter and enhancer sequences. 20 μ g of total RNA from tissues of one control and one homozygous 397-7 mouse was loaded per lane, transferred to Nytran membrane, and probed with a 32 P-labeled probe to the full-length K14.P sequence. Control heart, brain, kidney, and liver as well as transgenic heart, brain, and kidney had no detectable transgene mRNA expression whereas hybridization to a band at the predicted size of the transgene (arrow) was detected in the transgenic (line 397-7) liver. Ribosomal RNA markers are shown on the left. Bar: (b) 6 μ m.

liver (Fig. 2). Frozen sections were incubated with either an anti-K8 or K18 antibody and/or the substance P antibody. In control hepatocytes, anti-K18 labeling showed an extended keratin network distributed throughout the cytoplasm (Fig. 2 *a*). An increase of labeling intensity was typically found around bile canaliculi, a tubular space formed between adjacent hepatocytes that delineates the beginning of the bile duct system. Substance P antibody labeling was not detected in hepatocytes of control liver (Fig. 2 *b*). To examine the IF network of transgenic hepatocytes, double label immunofluorescence using the anti-K8 (rat monoclonal) and substance P (rabbit polyclonal) antibodies was carried out (Fig. 2, *c* and *d*). Double-labeled hepatocytes were abundant, with most hepatocytes showing some degree of network perturbation (Fig. 2, *c-f*). The degree of perturbation ranged in severity: some hepatocytes had filaments that appeared slightly fragmented (Fig. 2 *c* and *d*) whereas others had filaments that were totally collapsed in large aggregate structures found adjacent to the plasma membrane (Fig. 2, *e* and *f*). No apparent regionalization of transgene expression in the liver was observed.

While nearly all hepatocytes were double labeled for the K14.P and K8 proteins, some anti-K8-labeled cells (arrows, Fig. 2, *c* and *d*) did not express the K14.P protein, even in mice bred to homozygosity. This lack of labeling may represent hepatocytes that do not express transthyretin or may be other cell types (e.g., Kupffer cells, fat storing cells, and endothelial cells) that normally do not express transthyretin. Keratin networks in these non-K14.P expressing cells routinely appeared normal.

Histopathology of the K14.P Transgenic Liver

The histology of transgenic and control livers was examined using light level microscopy to determine whether K14.P expression induced abnormal changes. In general, no major alterations were detected in young mice (up to ~4 months old) when compared to controls. However, in many older transgenic mice (~75% in the 397-7 line), various degrees of histopathologic change had occurred that were not present in age-matched controls (Fig. 3 *a*). In some transgenics, marked degenerative changes characterized by ballooning and clear cell changes of the cytoplasm were a prominent feature and were associated with nuclear degeneration in some areas (Fig. 3 *b*). Changes in some samples appeared regionalized, being more severe in hepatocytes surrounding the central vein, whereas in others the entire lobe was involved. Numerous areas of inflammatory infiltration (lymphohistiocytic nodules) (Fig. 3, *c* and *d*) as well as focal punch-out necrosis were noted (Fig. 3 *e*). In some cases transgenic liver exhibited loss of adhesion at cell borders (Fig. 3 *f*). Three cases, of approximately sixty examined, exhibited neoplastic nodule formation (hepatocellular hyperplasia; Maronpot et al., 1986) in areas of marked cytoplasmic degenerative changes, especially where clear cell changes had occurred (Fig. 3 *g*). Focal necrosis and accumulation of small ductular-like cells that appeared to be oval cells were also recognized (Fig. 3 *h*). Oval cells are considered immature cells that undergo proliferation in livers of rodents exposed to carcinogens and may serve as liver stem cells (for review see Sell, 1990). Mallory body structures were not apparent in any of the liver tissues examined.

Liver plays an important role in glycogen metabolism and contains enzymes for its synthesis and storage. As needed, glycogen is broken down into free glucose and released into the blood. Glycogen content in transgenic mouse liver was evaluated by PAS staining and found to have accumulated at markedly increased levels (Fig. 4 *a*) when compared with age-matched control liver (Fig. 4 *b*). Glycogen was typically present in large clumps throughout the cytoplasm suggesting a defect in processing and/or secretion was present in the hepatocytes of transgenics. Lipid accumulation was also examined by staining frozen sections of control and transgenic livers with oil-red O (Fig. 4, *c* and *d*). Control livers typically showed little to no staining, where the majority of sections examined from older transgenics had a marked increase. Lipid accumulation by itself is considered a benign change, though it may represent a precursor to changes leading to conditions such as cirrhosis.

In some instances the histopathology observed in livers from older transgenics was indicated by visible white nodules on the surface of the liver. No other alterations on the gross level were consistently apparent. Liver to body weight ratios for transgenic and control livers showed no significant difference between the two groups (not shown).

Electron Microscopy Analysis of the TTR-K14.P Liver

Electron microscopic examination of the transgenic liver tissue showed alterations in ultrastructure from control liver (Fig. 5 *a*). As expected from the light level microscopic analysis, transgenic liver hepatocytes had extensive glycogen accumulation (Fig. 5 *b*). An increased number of fat vacuoles of various sizes was also apparent throughout the liver (Fig. 5 *c*). In some hepatocytes, clumps of dense, amorphous material near the plasma membrane were identified (arrows, Fig. 5 *d*). This material appears similar to aggregated keratin protein, though unequivocal identification cannot be made without further immunological analysis.

Light level histological analysis of transgenic liver revealed areas in which hepatocytes were less cohesive (Fig. 3 *f*). It was of interest therefore to examine desmosome contacts since this type of junctional complex has close spatial and functional connections with keratin filaments. Light level analysis of desmosome-keratin associations using double-label immunofluorescence was carried out on frozen liver sections labeled with the anti-K8 rat antibody and a rabbit polyclonal antibody directed against the desmoplakin I/II proteins (Pasdar and Nelson, 1988). Despite disruption of the keratin network and in some cases total loss of filaments from the cytoplasm, no significant redistribution in the DP I/II antigens was apparent in the transgenic hepatocytes (not shown). However, preliminary examination of two transgenic liver samples by electron microscopy did show abnormal desmosomal linkages between cells (Fig. 5 *e*). Desmosomes appeared separated from the adjacent cell at the plasma membrane, which would result in loss of cell adhesion.

Alteration of the Keratin Network Inhibits Bile Flow and Bile Acid Secretion

A major function of liver hepatocytes is to secrete bile, a digestive emulsifying solution composed of bile acids, lipids, proteins, anions, and cations (Nathanson and Boyer, 1991). Bile is formed by the transport of osmotically active

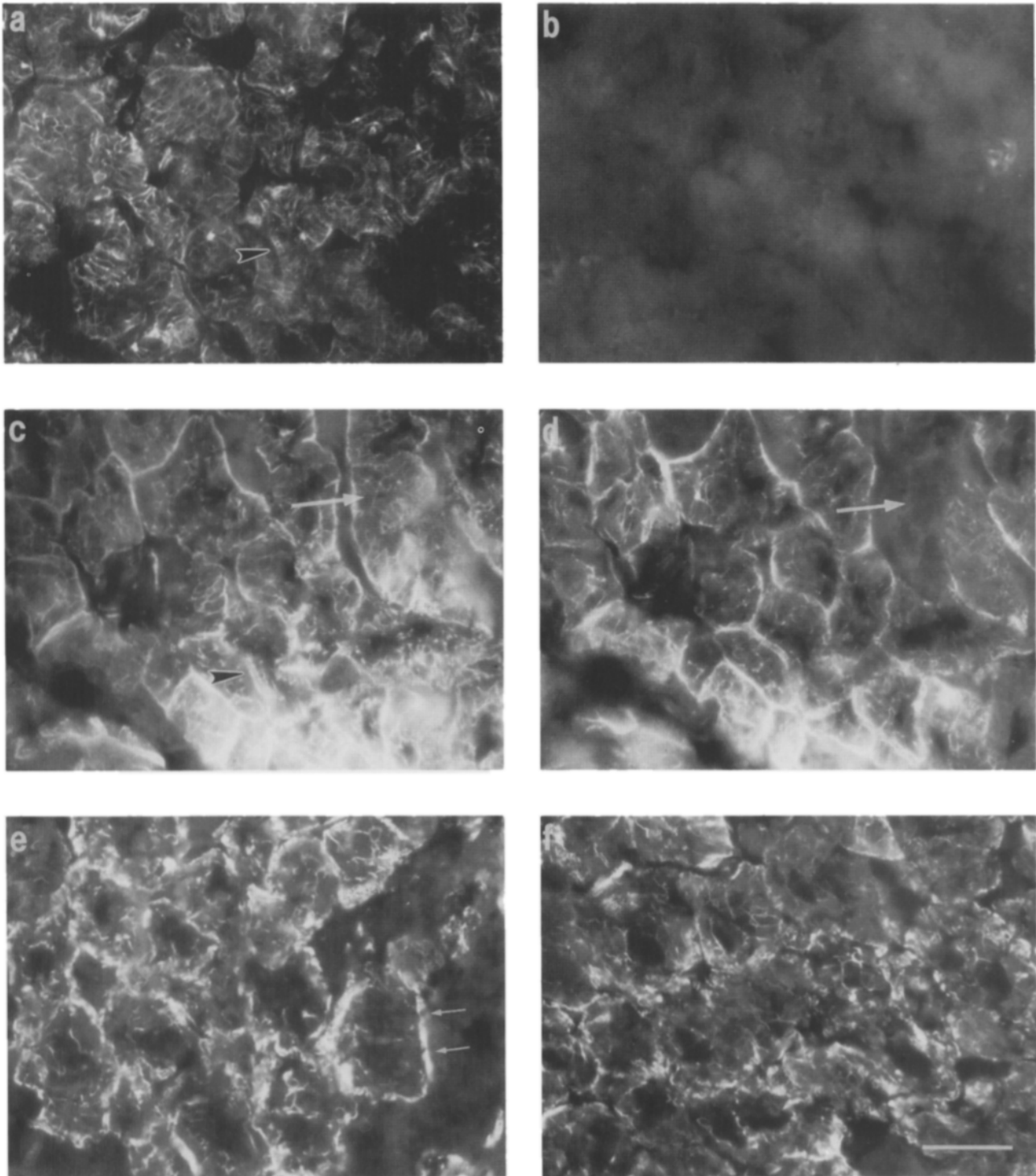


Figure 2. Transgene product expression in liver hepatocytes. Frozen sections of liver from control (*a* and *b*) and transgenic mice (*c*–*f*) were immunolabeled to detect keratin expression. Control liver was double labeled with the monospecific anti-K18 antibody (*a*) and the substance P antibody (*b*). Anti-K18 labeled the endogenous network and showed filaments extending throughout the hepatocyte with associations at the nuclear and cytoplasmic membranes. Arrowhead points to bile canaliculus. No reactivity was detected with the anti-SP antibody (*b*). Section from transgenic liver (*c* and *d*) double labeled with the troma 2 antibody (*c*) and the anti-SP antibody (*d*) to show overlapping expression of the K14.P keratin and the endogenous network. Note that filaments appear fragmented and shorter than in control tissue. Some cells (*arrows*) did not express the TTR-K14.P product. Arrowhead points to bile canaliculus. *e* and *f* show areas in the transgenic liver labeled with anti-SP where the filament network is more severely disrupted. Note intense labeling at plasma membrane and lack of filaments in cytoplasm. Sections were labeled with the anti-SP antibody followed by incubation with an FITC-conjugated IgG. Bar, 30 μ m.

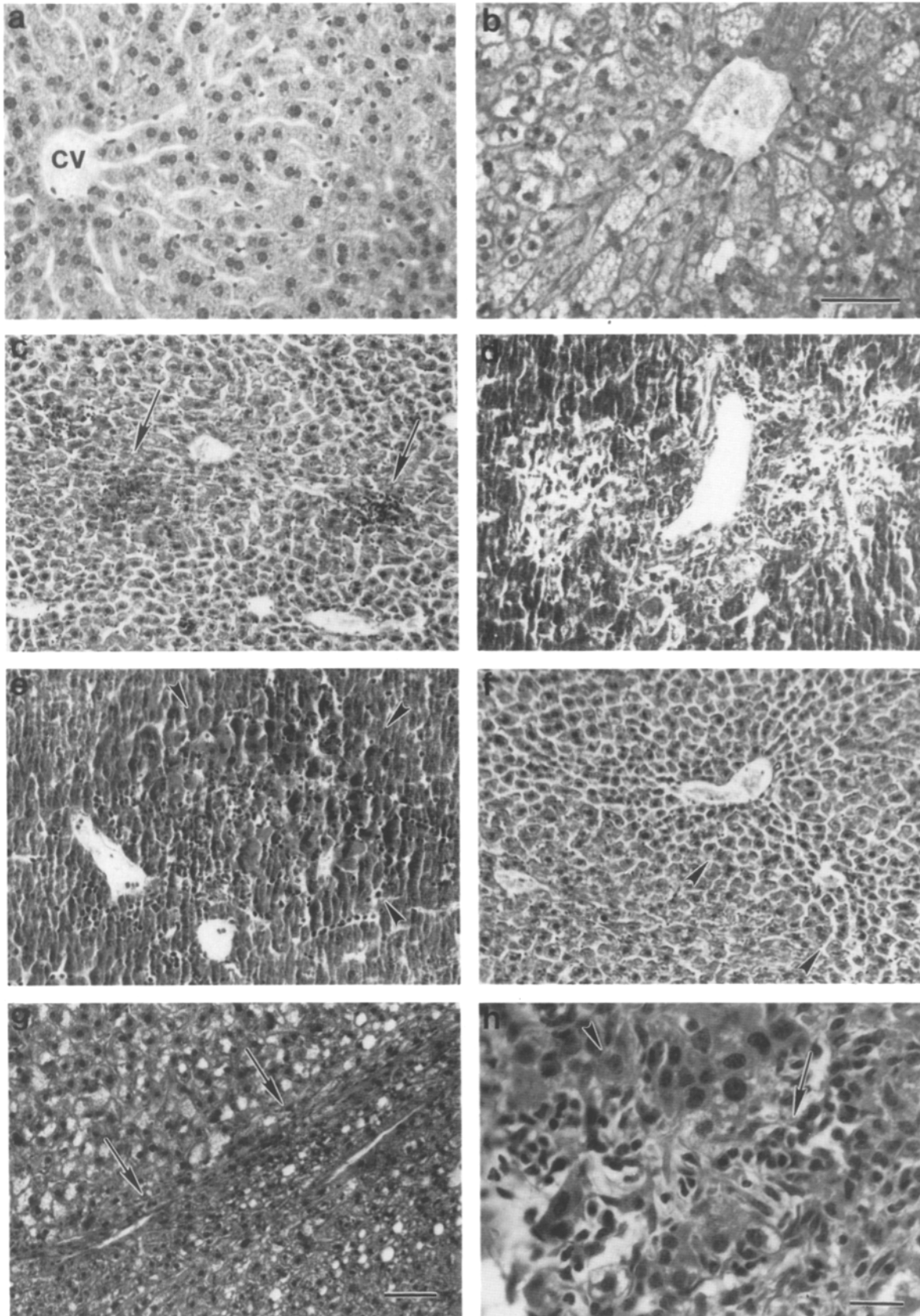


Figure 3. Histopathology of K14.P transgene expressing liver. To examine whether K14.P expression altered the histology of transgenic liver, tissue sections were fixed, processed, and stained with hematoxylin and eosin. (a) Control liver; (b) transgenic liver showing fatty and clear cell changes; (c) lower magnification illustrating islands of inflammatory cell infiltration (*arrows*); (d) a more advanced inflammatory infiltrate surrounding a vein; (e) area of focal necrosis demarcated by arrowheads. Note darker staining cells with pyknotic nuclei and inflammatory infiltrate; (f) field showing loss of cellular adhesion (*arrowheads*) primarily seen in central vein areas of some specimens; (g) transition zone marked by arrows between an area with clear cell changes to one containing a neoplastic nodule (hepatocellular hyperplasia); (h) higher magnification of a portal triad region that exhibited marked inflammatory infiltration and contained numerous oval cells. Arrows point to one area of oval cells; *arrowhead*, hepatocyte, *cv*, central vein. Bars: (b) 100 μm ; (g) 100 μm ; (h) 50 μm .

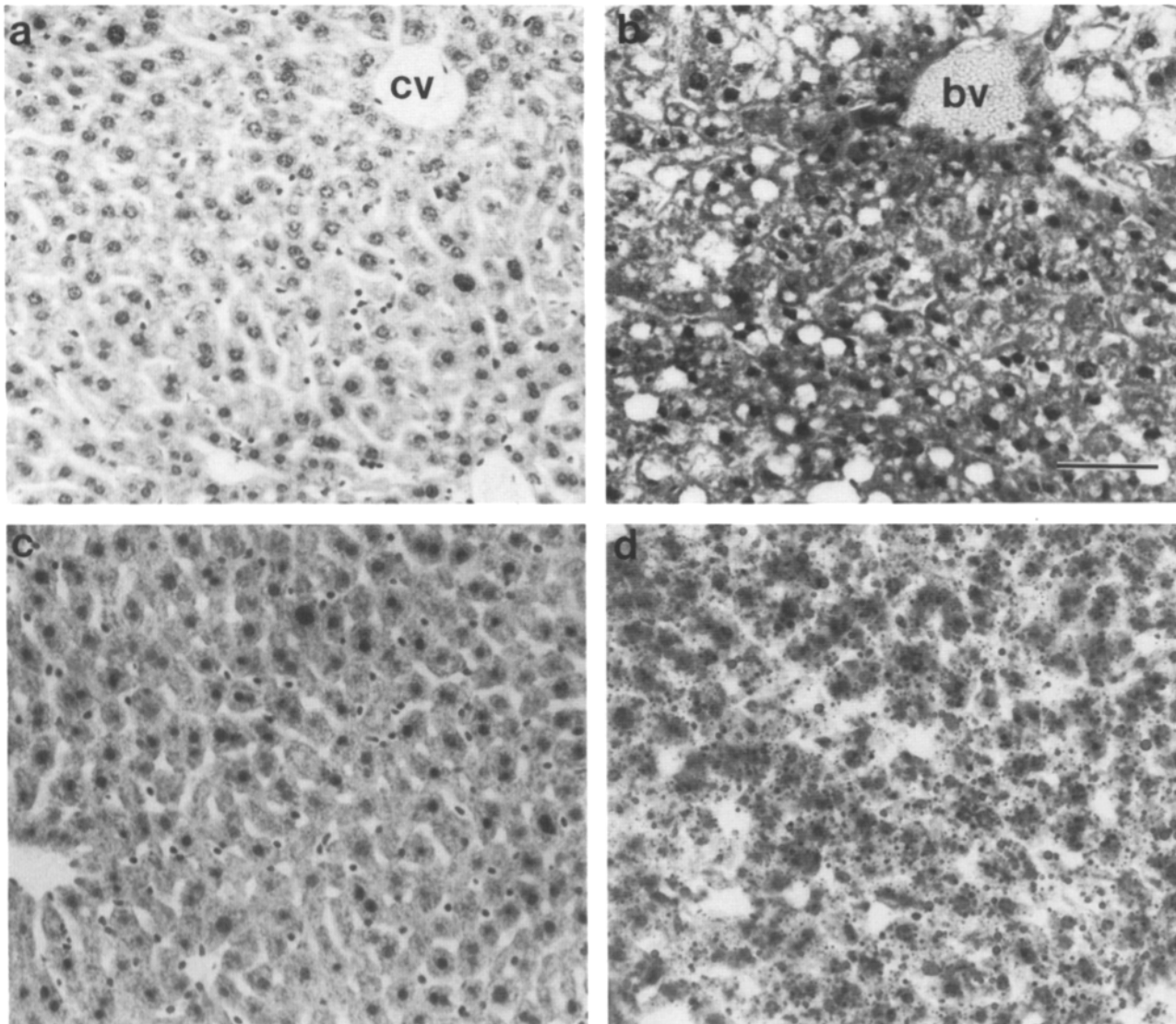


Figure 4. Transgenic livers accumulate glycogen and lipid. Overall glycogen accumulation was markedly increased in transgenic livers as shown using PAS staining. *a* shows control liver which typically had small amounts of glycogen staining in hepatocytes surrounding the central vein; *b* is liver tissue from transgenic showing marked increase in glycogen in nearly all hepatocytes in this field. Lipid was detected using oil-red O staining and was elevated in some, though not all, transgenic samples (*d*) when compared to controls (*c*). *cv*, central vein; *bv*, blood vessel. Bar, 100 μ m.

bile acids such as taurocholate across the basolateral (sinusoidal) domain of the plasma membrane to the apical (canalicular) domain. Flow into the bile canaliculus is followed by the passive diffusion of water until osmotic equilibrium is reached. To assess whether alteration of the keratin network affected bile secretory function, bile flow and bile acid secretion were measured in control and 397-7 and 482-4 transgenic mice. Tritium-labeled taurocholate (TC), a major component of bile, was used to measure the efficiency of a perfused liver to extract TC from the perfusate and secrete it into bile (Liu et al., 1992). Measures of taurocholate secretion from four 397-7 transgenic and five control livers showed the transgenics to have a 1.5–2-fold reduction in bile flow (μ l/min/g) (Fig. 6, *top*). The maximal bile acid secretory rate (SR_m) was also significantly decreased in transgenic mice relative to controls (Fig. 6, *bottom*). The concen-

tration of TC in secreted bile was unchanged in transgenics as compared to controls (Fig. 6, *middle*). Bile flow measures were also carried out on three 482-4 transgenics and age matched controls (not shown), and, as in the 397-7 line, had a 1.5–2-fold reduction in flow (transgenic_{avg} = 1.79; control_{avg} = 3.08) and a decrease in the SR_m (transgenic_{avg} = 191; control_{avg} = 369).

Discussion

We have shown using a transgenic mouse model system that the ectopic expression of an epidermal keratin in hepatocytes causes perturbation of the endogenous K8/K18 intermediate filament network, and can lead to histopathologic and functional abnormalities of the liver. These abnormalities included cytoplasmic degeneration in hepatocytes, infiltration

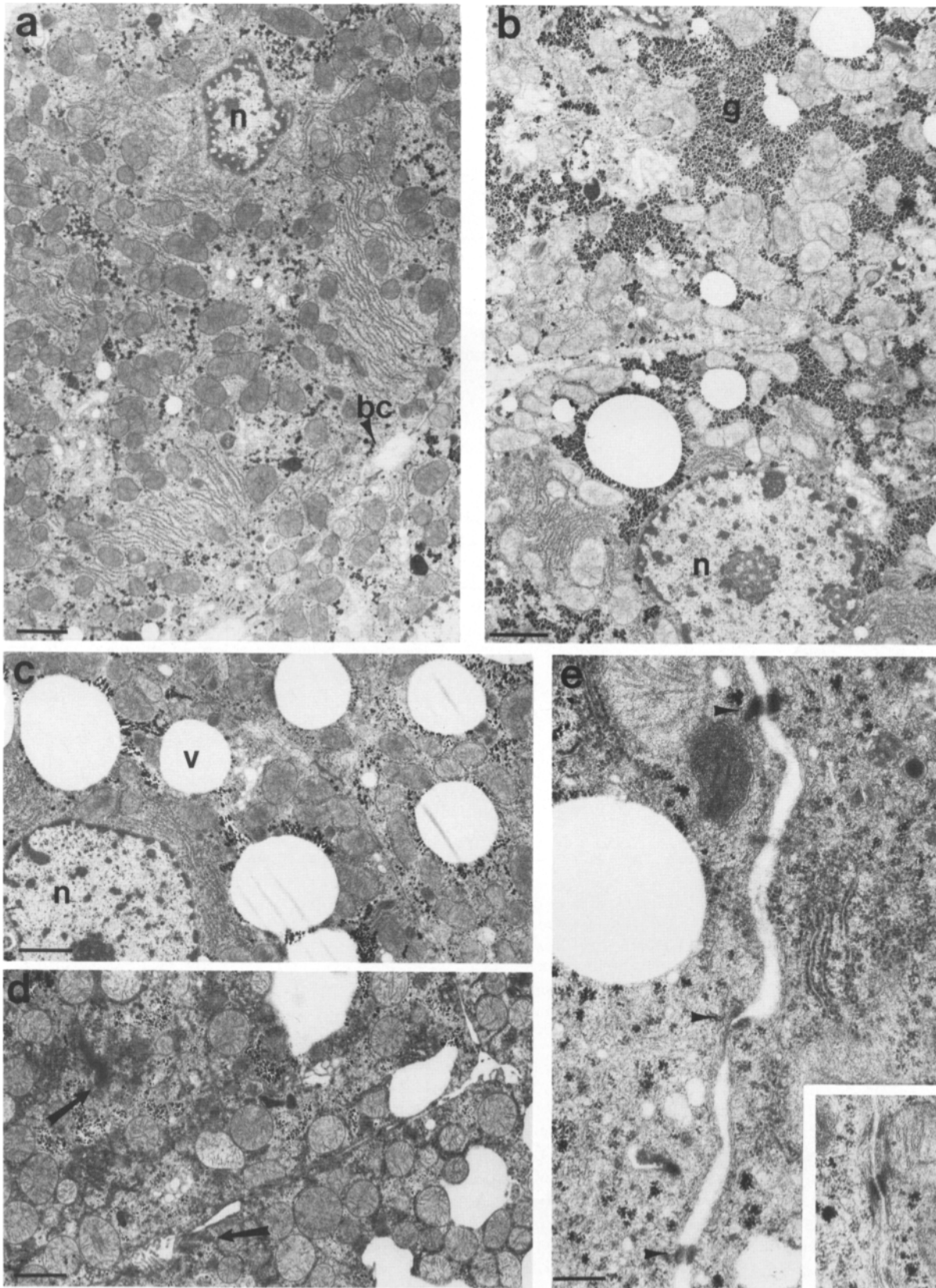


Figure 5. Electron microscopy analysis of transgenic liver. The ultrastructure of the transgenic livers was examined by electron microscopy and showed alteration in structure from control liver shown in *a*. *b* illustrates the increased amounts of glycogen (*g*) accumulated in the cytoplasm of hepatocytes. *c* shows a portion of a hepatocyte with numerous fat-containing vacuoles. This field shows medium-sized vacuoles, though small and large vacuoles were also apparent. Arrows in *d* point to dense material that may be aggregated keratin protein, similar to that detected on the light histology level by immunolabeling with anti-substance P antibody (see Fig. 2 *e*). *e* shows an abnormally wide junction between two hepatocytes. Arrowheads point to desmosome structures that appeared separated. *n*, nucleus; *bc*, bile canaliculus; *g*, glycogen; *v*, vacuole. Magnifications are: (*a*) 6,000; (*b*, *c*, *d*) 5,500; (*e*) 29,000. Bars: (*a*) 4.7 μm ; (*b*, *c*, and *d*) 2.5 μm ; (*e*) 0.45 μm .

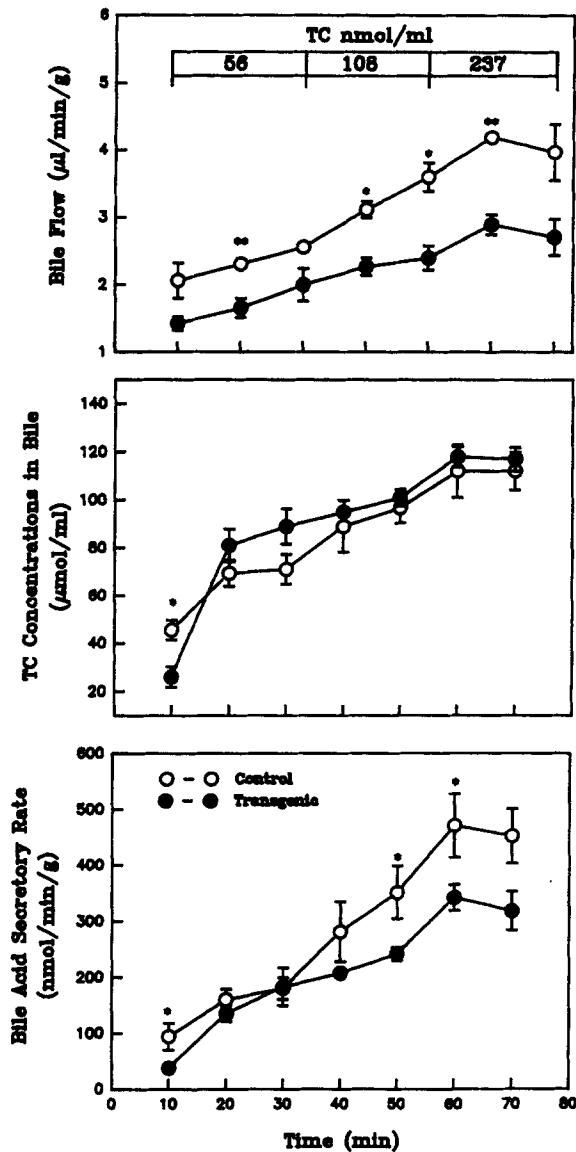


Figure 6. Bile flow measures in transgenic and control mouse liver. To evaluate secretory function in the TTRK14.P liver, bile flow (top), bile acid concentration (middle), and bile acid secretory rate (bottom) in four 397-7 transgenic and five age and sex matched control livers were measured. Each point is the mean \pm standard error. * $p < 0.05$; ** $p, 0.01$, significantly different from control group. Similar results were obtained using mice in the 482-4 line.

of inflammatory cells, fatty accumulation, glycogen accumulation, hepatocellular hyperplasia, and decreased bile acid flow. Although the underlying cellular mechanism of these disturbances is not as yet understood, an increase in the severity of the histopathology occurred with age, indicating that progressive damage to the hepatocyte ultimately led to the degeneration in liver function. The observed histopathologic findings are not specific for, but may be seen with, repeated toxic injury to the liver. Therefore, this transgenic animal model may provide a useful tool for studying pathogenesis as well as the tumorigenic effect of toxin induced liver disease.

Livers of the transgenic mice displayed cellular and functional abnormalities even though transfection of the TTR-

K14.P plasmid into cultured hepatocytes caused no obvious alteration in the keratin network. Thus, the composition of the network appears to be more crucial in the context of the tissue than in transfected cells grown in plastic, i.e., hepatocytes in vivo may require a keratin network finely tuned to the metabolic and secretory functions of the liver, challenges not presented to cells in culture. This contention is in agreement with the findings of Blessing and colleagues (1993) who showed that ectopic expression of either the type II K1 epidermal keratin alone or in combination with its type I K10 partner caused an insulin-dependent form of diabetes mellitus characterized by reduction in β islet cells and a massive reduction of insulin. Pancreatic islet cells that overexpressed either K1 alone (but not K10) or in conjunction with K10 keratin had accumulated fibrous material and a reduction in the number of insulin-secretory vesicles. What was surprising in these studies was that the K10 keratin when expressed alone, even in large excess, did not affect islet cell secretion. The basis for this apparent keratin specificity to perturbation of islet cell function is not known. Even so, these results and our findings in the TTR-K14.P transgenics suggest that the keratin filament network may play a role in secretion.

To assay liver function in the TTR-K14.P transgenics, bile acid production was measured. Bile secretion represents an important route for the elimination of both endogenous and exogenous organic compounds such as cholesterol, bile salts, drugs, and their metabolites, which cannot be easily removed by the kidney from the body (Nathanson and Boyer, 1991). Hepatocytes behave as polarized epithelial cells and transport bile secretory products from the sinusoidal to the canalicular domain. The cytoskeletal components of the hepatocyte are thought to have an important role in bile acid uptake, transport, and secretion. Microtubules form a dense network near the sinusoidal membrane and from studies using microtubule disruptors, appear to function in vesicular internalization and transport through the cytoplasm (Goltz et al., 1992; Feldman, 1989). Microfilaments are densely arranged at the canalicular membrane and may mediate coordinated contractions between hepatocytes to facilitate bile flow (Oshio and Phillips, 1981; Smith et al., 1985). Keratin intermediate filaments also have a close association with the canalicular membrane, though their role in bile acid uptake, transport, and secretion has been difficult to evaluate. Disruption of keratin filaments in cultured hepatocytes was achieved by exposure to nickel chloride and caused decreased uptake of applied horseradish peroxidase, an inability to secrete the organic anion fluorescein diacetate, and dispersion of bile canalicular structures (Kawahara et al., 1990). These alterations in secretion may have resulted from a direct perturbation of the keratin network, though another possibility is that the phenotype was induced by a less specific, nickel-based toxicity.

The cellular location at which bile acid secretion is impeded in the transgenics is unknown; it may occur during uptake, transcytosis, secretion, or may be slowed in all three domains. Because transport of taurocholate across the sinusoidal domain normally exceeds that across the canalicular domain by sixfold (Nathanson and Boyer, 1991), it seems more likely that the defect occurs at the canalicular membrane, the rate-limiting step. Another possibility is that bile flow in transgenic hepatocytes is physically impeded due to the increased number of fat vacuoles and glycogen deposits.

Bile flow measures were done on eight month-old mice, an intermediate age at which some degree of histopathologic alteration could be present. Further studies measuring flow rates in young transgenics, typically without overt histopathology, and older mice should clarify this issue.

A common feature observed in degenerative diseases of the liver is the formation of Mallory bodies. Mallory body structures are amorphous in nature and contain bundles of intermediate filaments organized in various arrays (Franke et al., 1979; Denk et al., 1982; French et al., 1987; Yokoo et al., 1972). They can be induced in mouse liver by prolonged feeding of griseofulvin and are typically arranged in prominent perinuclear arrays (Denk et al., 1979). Though Mallory bodies are associated with a number of hepatic disorders, the conditions that lead to their development are not understood. Since expression of the K14.P keratin caused perturbation in the hepatocyte IF network, it was possible that Mallory bodies would develop in the transgenic livers. However, examination of transgenic hepatocytes on the light and electron microscope level showed no evidence of typical Mallory body structures. Cells in the transgenic liver with abnormal keratin networks had filaments that were fragmented and collapsed to the plasma membrane. Thus, the collapsed keratin networks in the transgenic liver appear to be induced by a separate mechanism from that involved in the formation of mature Mallory bodies. It may be that other components or physiological conditions are required for their formation.

Although a function for IFs as a group remains undefined (Oshima, 1992), studies using transgenic mice that express mutated keratin proteins in skin have demonstrated their crucial role in maintaining keratinocyte structure and imparting mechanical integrity to the skin (Fuchs, 1994). Transgenic mice that expressed truncated or site mutated keratin genes exhibited keratin filament clumping and resultant blistering of the skin (for review see Coulombe, 1993). Gene mapping and DNA sequence studies have shown that patients with the dominantly inherited forms of epidermolysis bullosa simplex (EBS) and epidermolytic hyperkeratosis (EH) blistering diseases have defective keratin proteins resulting from point mutations in epidermal keratin gene sequences (Coulombe et al., 1991; Bonifas et al., 1991; Lane et al., 1992; Chan et al., 1993). Disease severity has been correlated with the relative importance of the affected amino acid residue(s) to this filament structure, i.e., mutations in highly conserved residues caused a severe blistering phenotype whereas those in less conserved residues led to milder phenotypes (Letai et al., 1993). Interestingly, EH is characterized by diminished cohesiveness between keratinocytes, similar to the reduced cohesiveness observed in some of the TTR-K14.P liver specimens. Keratinocytes with perturbed keratin networks also displayed abnormalities in organelle and nuclear placement, suggesting the network serves as a cellular scaffold (Coulombe et al., 1992). Hepatocytes are likely to have a similar scaffold organization that if perturbed could disrupt normal cellular organization.

Studies of simple epithelial function in genetically modified embryonic stem cells have shown that the K8 keratin is not required for the early phases of mouse development; K8 deficient ES cells differentiated into embryoid bodies composed of an outer polarized simple epithelium and internal primary ectoderm that developed normally (Baribault and Oshima, 1991; but see Trevor, 1990). Thus,

at early time in development keratin networks may be non-essential. In further examination of the role of keratins, Baribault and colleagues (1993) isolated gene knockout mice containing a null mutation in the mouse K8 gene (*mK8*^{-/-}). Homozygous mutant embryos were growth retarded, and, though some survive to adulthood, most die between E12 and E13, possibly from internal bleeding. During this time fetal hematopoiesis shifts from the yolk sac to the liver and it may be that the keratin filament deficient fetal liver was unable to accommodate the massive increase in hematopoiesis (Baribault et al., 1993). Interestingly, other studies by H. Baribault and colleagues (1994) on *mK8*^{-/-} survivors show that some of these mice exhibit inflammatory cell infiltration and slight elevation in levels of the hepatic enzymes alanine (AST) and aspartate (ALT) aminotransferase, findings typically associated with hepatocyte injury. This suggests that lack of K8 and thus the hepatocyte IF network, has consequences on liver function.

In interpreting the transgenic phenotype, our working hypothesis has been that the observed alterations are in some way linked to the perturbation of the keratin filament network. However, another possibility to explain these changes is that they result from an accumulation of an abnormal protein (K14.P) in hepatocytes that, over time, leads to defects in cellular structure and function. Though expression of the transgene protein is low relative to the endogenous IF proteins, sufficient amounts of the insoluble K14.P protein may have accumulated in the cytoplasm to cause a nonspecific toxicity. Another issue is whether the general low level of transgene expression across lines resulted from low promoter activity or perhaps embryonic lethality induced by high level of transgene protein. No significant increase in the number of dead pups in founder litters was observed, though it is possible they died in utero.

We have used transgenic mice that express an epidermal keratin derivative in liver hepatocytes to examine the role of keratin proteins in cellular and tissue function. Though K14.P formed normal appearing networks in cultured liver cells, in the context of the tissue its presence caused network abnormalities and changes in liver histology and function. This model may have importance in understanding the myriad of liver disorders in which keratin filament structure and/or composition is abnormal.

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