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Complete reconstitution of mouse liver with xenogeneic hepatocytes

(liver cell transplantation/liver regeneration/xenogeneic transplantation/transgenic)

JONATHAN A. RHIM*†, ERIC P. SANDGREN*‡, RICHARD D. PALMITER§, AND RALPH L. BRINSTER*¶

*Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104; †Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; and §Howard Hughes Medical Institute and Department of Biochemistry, University of Washington SL-15, Seattle, WA 98195

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ABSTRACT **We have developed a system for studying hepatocellular growth potential in which liver cells are introduced into the diseased livers of albumin–urokinase (Alb-uPA) transgenic mice. To use this system to study xenogeneic cell transplantation, rat liver cells were introduced into immunotolerant Alb-uPA transgenic mice. In regenerated recipient livers, up to 100% of hepatocellular gene expression was of rat origin, demonstrating the creation of a functional mouse liver in which parenchyma is derived from xenogeneic (rat) hepatocytes. Immunotolerant Alb-uPA transgenic mice provide a tool for studying hepatocellular biology of any species, including humans, in a controlled experimental setting.**

We have developed a transgenic mouse system to assess the regenerative capacity of hepatocytes. In this system, albumin–urokinase (Alb-uPA) transgenic mice are recipients of donor mouse hepatocytes. The transplanted hepatocytes grow within the Alb-uPA liver (1), replacing transgene-expressing hepatocytes that are functionally compromised by transgene expression (2). Using this approach, we demonstrated that hepatocytes from adult liver have extensive replicative potential (1). The growth of the transplanted cells was nodular and clonal and occurred over several weeks. The resulting fully regenerated livers were chimeric, composed both of donor-derived cells and of host-derived cells that had deleted transgene DNA and therefore no longer expressed the transgene. Like transplanted hepatocytes, these host-derived cells also had a growth advantage relative to transgene-expressing hepatocytes and expanded in a nodular fashion (termed "red nodules"), competing with the donated hepatocytes. In hemizygous transgenic animals, transgene inactivation is a relatively frequent event, often resulting in hundreds of clonal nodules in young hemizygous mice (2); complete replacement of the transgenic liver by the clonal expansion of these nodules occurs by 8 weeks of age. By comparison, in homozygous mice, transgene inactivation is a less common event, consistent with the fact that two transgene arrays must be inactivated. Clonal nodules are rare in young homozygotes and, as a consequence, transgenic liver persists longer. Thus, hepatocyte transfer into homozygous Alb-uPA mice would be ideal for assessing liver cell growth; the lack of competition from red nodules should allow the donated cells to completely replace the recipient liver.

Because Alb-uPA transgenic mouse liver supported the growth of transplanted mouse hepatocytes (1), we hypothesized that immunotolerant mice would support the growth of transplanted hepatocytes from other species. For initial studies of xenogeneic cell transplantation, we chose to donate liver cells from the rat, the species most often used to study liver growth and development. Rat liver cells present several advantages as donor cells. Protocols exist to produce well-defined liver cell populations, which could be separately transplanted into Alb-uPA transgenic mice. The ability of each population to repopulate diseased recipient mouse liver would indicate both the replicative and lineage potentials of each class of cell. Furthermore, transplantation of nonhepatocytic cells into Alb-uPA transgenic mice might enable us to detect and to characterize hepatic stem cells, the existence of which remains controversial (3–5). Finally, if this method were feasible, Alb-uPA transgenic mouse livers repopulated with xenogeneic hepatocytes would be valuable tools for studying liver biology of other species, including humans, in a controlled, *in vivo* experimental setting.

MATERIALS AND METHODS

Generation of Immunotolerant Alb-uPA Transgenic Mice. To generate immunotolerant Alb-uPA transgenic mice, we crossed Alb-uPA transgenic mice (2) with Swiss athymic, nude (*nu/nu*) mice (Taconic Farms). Transgenic mice were identified by dot blot hybridization of tail DNA as described (2). Hemizygous transgenic *nu/nu* mice and homozygous transgenic *nu/nu* mice were generated by breeding hemizygous transgenic *nu/+* females with hemizygous transgenic *nu/nu* males. Initially, homozygous mice were distinguished from hemizygous mice by quantitative dot hybridization by measuring the ratio of radioactive probe annealed to duplicate dots after hybridization to either a transgene probe (800-bp *Bgl* II–*Eco*RI fragment from the human growth hormone gene) or an endogenous gene (4-kb fragment from the *Hoxa* locus). Subsequently, we took advantage of the observation that insertion of the transgene resulted in a deletion of endogenous DNA at the site of integration. An easier test of homozygosity was devised in which duplicate dots were hybridized with a transgene-specific probe (fragment from the human growth hormone gene) (as above) or a unique probe (550-bp *Bam*HI fragment) derived from the deleted region.

Liver Cell Isolation and Transplantation. Rat liver cells were isolated from 3- to 6-week-old female Sprague–Dawley rats (Taconic Farms) by two-step EDTA/collagenase perfusion using a protocol modified from Klaunig *et al.* (6). In the isolation procedure, liver cell suspensions were centrifuged twice at 50 × *g* to enrich for hepatocytes. Cell viability was determined by trypan blue exclusion and ranged from 50% to 90%. After isolation, liver cells were kept on ice and transferred within 2 hr. Liver cells ($1–2 \times 10^5$) were transplanted into recipient mice between 10 and 15 days of age by intrasplenic injection as described (1).

Detection of Rat-Specific DNA and mRNA. Rat and mouse DNAs were distinguished using a restriction fragment length

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Abbreviations: Alb-uPA, albumin–urokinase transgenic mice; mAb, monoclonal antibody.

‡Present address: Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706.
¶To whom reprint requests should be addressed.

polymorphism in the phenylethanolamine *N*-methyltransferase gene. Genomic DNA (6 μg) was digested with *Pst* I, electrophoresed through a 0.7% agarose gel, transferred to nitrocellulose, and hybridized with a 449-bp probe specific to exon 3 of the mouse phenylethanolamine *N*-methyltransferase gene (7). Fragments of different sizes were seen in rat and mouse control livers. In samples containing both rat and mouse DNA, the relative amount of rat DNA was calculated from the intensity of the radioactivity hybridized (determined using a Molecular Dynamics PhosphorImager) to the rat and mouse *Pst* I fragments using a standard curve generated by mixing various amounts of mouse and rat DNA. A 10% contribution of rat DNA was detectable.

Mouse and rat transferrin mRNAs were measured by solution hybridization using ^{32}P end-labeled oligonucleotides complementary to sequences in the 3′-untranslated regions of the mRNAs (8). The mouse probe (no. 415) was 5′-AAGGCACAGCAGCGAAGACTACAC-3′, which differs in eight positions (underlined) from the rat probe (no. 422), which was 5′-AACACACAGCAGTGAAGACGGACA-3′. The rat sequence was derived from our own sequence of the 3′-untranslated region of rat transferrin cDNA, which differed substantially from the published sequence. The hybridization mixture contained 10% (vol/vol) formamide and about 10,000 cpm of either probe. After incubation for ≈20 hr at 45°C, the nonhybridized probe was eliminated with S_1 nuclease, and the protected probe was precipitated with trichloracetic acid and collected on a Whatman GF/C filter for scintillation counting. Standard curves were made from normal mouse and normal rat liver nucleic acids. There was no cross-hybridization of the mouse probe with rat transferrin mRNA or vice versa. The sensitivity of the assay was sufficient to detect a 1% contribution of rat transferrin mRNA in a mouse liver sample.

Immunohistochemistry. Indirect immunostaining of liver sections was adapted from ref. 9. Mouse monoclonal antibody (mAb) 258.26 is reactive with rat hepatocytes; mAb 18.11 is reactive with rat bile ducts (10). The secondary antiserum was affinity-purified goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (Sigma). Frozen sections, 10 μm thick, were air-dried and fixed for 10 min in 100% acetone at 4°C. Sections were then washed in phosphate-buffered saline (PBS) and incubated with the primary antibody for 45 min at room temperature. The sections were then washed in PBS, incubated in 1% goat serum for 5 min, and incubated with a 1:100 dilution of the secondary antiserum for 45 min at room temperature. The sections were washed for 20 min in PBS and then incubated for 2 min with the chromogen 3,3′-diaminobenzidine. The sections were then lightly stained with Mayer's hematoxylin. Parallel sections stained only with the secondary antibody were negative (data not shown). Immunohistochemical detection of rat hepatocytes was confirmed using another rat-specific hepatocyte antibody, mAb 362.50 (11) (data not shown).

Liver was immunostained for α_{2u}-globulin protein based upon the procedure described in ref. 12 using the IgG fraction of a polyclonal rabbit antiserum to α_{2u}-globulin (a generous gift of A. Roy, University of Texas Health Science Center at San Antonio). Tissues were fixed in neutral-buffered formalin and embedded in paraffin for sectioning. Slides were deparaffinized and blocked with 3% (vol/vol) swine serum for 30 min, followed by incubation with the primary antiserum for 30 min at room temperature. Sections were washed in PBS. The primary antibody was detected using a peroxidase-antiperoxidase kit (Dako) according to the manufacturer's instructions. Treated sections were incubated with 3,3′-diaminobenzidine for 2 min and lightly counterstained in Mayer's hematoxylin. Parallel sections incubated with only secondary antibody were negative (data not shown). Control rat liver was taken from male rats ≈65 days of age.

RESULTS

Repopulation of Alb-uPA Transgenic Mouse Liver with Rat Hepatocytes. Rat liver cells were transplanted into the livers of immunotolerant (*nu/nu*) Alb-uPA transgenic mice that were either hemizygous or homozygous for the transgene. Unless otherwise specified, recipient mice were killed, and their livers were analyzed when liver regeneration was nearly complete—that is, when the liver was <10% transgenic by gross inspection. [Transgene-expressing liver had a characteristic pale white gross appearance (2), making it readily distinguishable from nontransgenic liver.] For hemizygous animals, this occurred between 6 and 8 weeks of age; for homozygous animals, this occurred between 10 and 14 weeks of age. At the time transplanted livers were examined, the recipient mice were clinically healthy (one homozygous mouse died at 10 weeks of unknown causes) and indistinguishable from nontransgenic *nu/nu* mice maintained in our colony. Serum albumin and total protein levels for both hemizygous and homozygous transgenic recipients were similar to transgenic nontransplanted and nontransgenic controls (data not shown).

Growth of transplanted rat liver cells could be detected in homozygous transgenic livers by gross inspection (Fig. 1) because of differences in color. At 8 weeks of age, homozygous transgenic livers that had not been transplanted with rat liver cells were completely pale in color, or "white" (Fig. 1*A*). In contrast, homozygous transgenic livers transplanted with rat liver cells were white and red (Fig. 1*B*); the latter resembled normal liver parenchyma (Fig. 1*C*), suggesting that these areas were composed of donor-derived rat liver cells. Red areas were multifocal and often nodular in shape, suggesting that numerous transplanted rat liver cells had engrafted and had begun to grow in the same nodular fashion as transplanted mouse liver cells (1). Completely regenerated homozygous transgenic livers resembled normal mouse livers in color, shape, and size (Fig. 1*D*). In hemizygous livers transplanted with rat liver cells, the growth of rat-derived cells was not discernible by gross

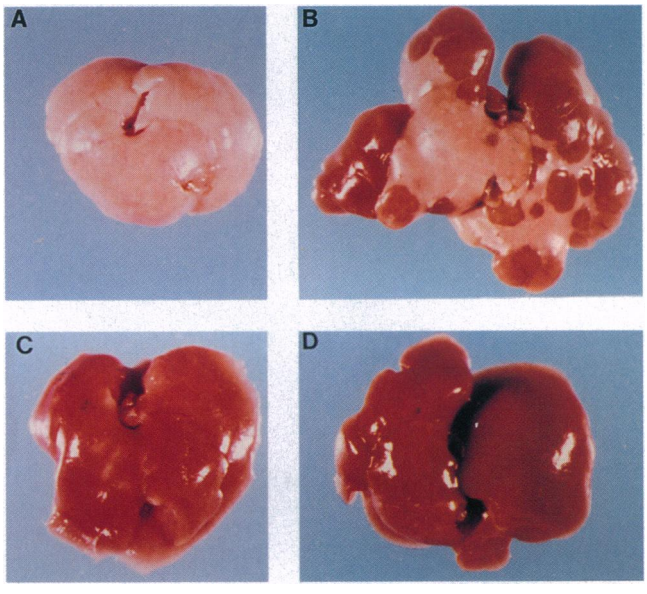

FIG. 1. Liver regeneration in homozygous transgenic *nu/nu* mice transplanted with rat liver cells. (*A*) Nontransplanted, homozygous transgenic control liver (8 weeks). The liver is uniformly pale in color, or white, due to the expression of the hepatotoxic transgene (2). (*B*) Partially regenerated homozygous transgenic *nu/nu* liver transplanted with rat liver cells (8 weeks). The partially regenerated liver contains white areas and nodular areas that are the color of normal liver (i.e., red). (*C*) Nontransgenic control mouse liver. (*D*) Completely regenerated homozygous transgenic *nu/nu* liver transplanted with rat liver cells. This liver is similar in color, shape, and size to the control mouse liver shown in *C*.

inspection (data not shown) because rat-derived liver resembled mouse liver derived from transgenic hepatocytes that had inactivated the transgene (2). The liver-to-body weight ratio of transgenic mice transplanted with rat liver cells was similar to that of nontransgenic control mice (6.8% ± 1.0%, n = 16; and 5.8% ± 0.6%, n = 10, respectively).

Rat DNA was detected in hemizygous and homozygous mouse livers by Southern blot hybridization (Fig. 2). Restriction fragments of different sizes were detected when mouse versus rat DNA was digested with *Pst* I and hybridized to a probe specific to exon 3 of the mouse phenylethanolamine *N*-methyltransferase gene. The rat-specific restriction band was detected within regenerated liver from both hemizygous and homozygous transplant recipients (Fig. 2). In hemizygous liver samples, the intensity of the rat DNA band varied considerably, ranging from undetectable to comparable to that of homozygous liver samples (Fig. 2). Quantitation of the Southern blot DNA bands demonstrated that up to 56% of the DNA in some liver samples was of rat origin.

Rat hepatocytes were detected immunohistochemically within transplanted livers using a mAb that did not stain mouse hepatocytes (Fig. 3*A*) but strongly stained rat hepatocytes (Fig. 3*B*). No staining of bile ducts or vessels was evident (Fig. 3*B*). Regenerated hemizygous livers that had been transplanted with rat liver cells showed stained and unstained areas (Fig. 3 *C* and *D*). The unstained areas, which were nodular in shape, were most likely composed of mouse hepatocytes that had inactivated the transgene (1). Regenerated homozygous liver showed extensive staining (Fig. 3 *E* and *F*), suggesting that it was composed predominantly of rat hepatocytes. Unstained areas were seen within regenerated homozygous liver around portal tracts, suggesting that these areas were composed of residual host-derived cells (Fig. 3*F*). The biliary epithelium within portal tracts did not react with a mAb specific for rat bile ducts, suggesting that the biliary tree was of mouse origin (data not shown).

Detection of Rat Gene and Protein Expression in Rat–Mouse Chimeric Livers. To detect rat gene expression in the rat–mouse chimeric livers, we analyzed transferrin gene transcripts using probes that could distinguish between rat and mouse transcripts; rat transferrin mRNA levels were expressed as a percentage of total transferrin mRNA detected (rat plus mouse). Transferrin mRNA analysis was performed on randomly selected samples of regenerated (i.e., red) liver from hemizygous and homozygous transgenic recipients. In regenerated liver samples from hemizygous recipients, the percentage of transferrin transcripts of rat origin varied considerably, ranging from <1% to 92% (n = 12). In regenerated liver samples from all three homozygous animals analyzed, 90–100% of the transferrin transcripts were derived from the rat (n = 14). The average percentages ± SD for each of the three homozygous mice were 98.5% ± 2.1%, n = 2; 98.5% ± 0.9%, n = 3; and 96.1% ± 4.7%, n = 9. Lower levels of rat transferrin mRNA were seen in those areas of liver containing residual white, nonregenerated liver. For example, one area of predominantly white liver from the specimen shown in Fig. 1*B* showed 70% rat transferrin mRNA. (The origin of the rat transcripts in this specimen was unclear, although they were presumably from contaminating rat hepatocytes). The reproducibly high repopulation of homozygous transgenic liver by rat liver cells is consistent with the fact that homozygous animals, unlike hemizygous animals, do not generate many mouse-derived red nodules.

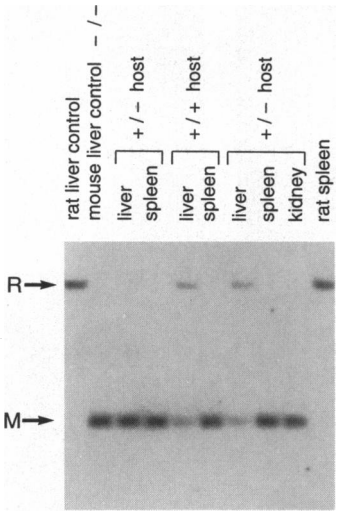

FIG. 2. Southern blot analysis of regenerated liver from transgenic *nu/nu* mice transplanted with rat liver cells demonstrating the presence of rat DNA. Liver samples were taken from regenerated areas of hemizygous (+/−) and homozygous (+/+) transgenic *nu/nu* mouse livers transplanted with rat liver cells and analyzed for rat DNA by Southern blot hybridization. Fragments of different sizes were detected in rat (R) and mouse (M) control livers. In transgenic liver samples containing both mouse and rat fragments, note the diminished intensity of the mouse liver fragment relative to spleen. −/−, Nontransgenic; +/−, hemizygous for the transgene; +/+, homozygous for the transgene.

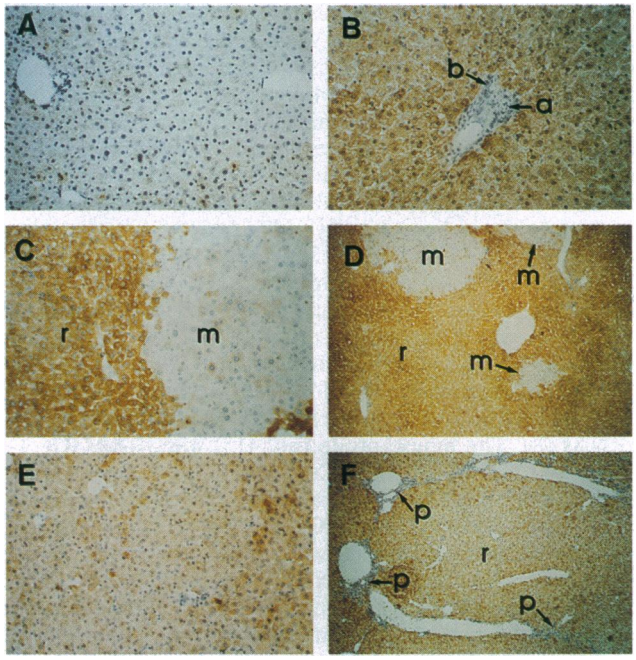

FIG. 3. Immunohistochemical detection of rat hepatocytes in regenerated hemizygous and homozygous transgenic *nu/nu* mouse livers transplanted with rat liver cells. (*A–F*) Frozen sections of liver immunostained with a mAb against rat hepatocytes and counterstained with hematoxylin. (*A*) Control nontransgenic mouse liver. Occasional hepatocytes are lightly stained. (*B*) Control rat liver. All hepatocytes are strongly stained. An arteriole (a) and bile duct (b) within a portal tract are not stained. (*C*) Hemizygous transgenic *nu/nu* mouse transplanted with rat liver cells. Rat-derived (r) stained areas and mouse-derived (m) unstained areas are evident. At the boundary between the two areas, stained and unstained hepatocytes intermingle, without a sharp boundary. No compression of cells, as is often seen in expanding tumors, is evident. (*D*) Hemizygous transgenic *nu/nu* mouse transplanted with rat liver cells. The unstained, mouse-derived (m) areas are nodular in configuration and are separated from one another by areas of rat (r) cells. (*E*) Homozygous transgenic *nu/nu* mouse transplanted with rat liver cells. All cells are stained. (*F*) Homozygous transgenic *nu/nu* mouse transplanted with rat liver cells. Unstained portal areas (p) are grouped around large areas composed of rat hepatocytes (r). (*A–C* and *E*, ×75; *D* and *F*, ×40.)

The percentage of rat mRNA detected in recipient livers showed a linear correlation ($r = 0.9757$) with the percentage of rat DNA detected (Fig. 4). As the rat transferrin mRNA detected in liver samples approached 100%, the rat DNA approached 60%, the percentage of hepatocytes in normal liver. No rat transferrin mRNA was detected with the rat probe in either nontransgenic *nu/nu* livers or in nonimmunosuppressed (non-*nu/nu*) transgenic livers transplanted with rat liver cells (data not shown).

Rat protein expression was detected by immunohistochemistry using antiserum to the rat liver protein, α_{2u}-globulin (12). In the rat, α_{2u}-globulin is synthesized by hepatocytes, secreted into the blood, freely filtered by the kidney, and reabsorbed by renal tubular epithelial cells (13, 14). In normal rat liver, α_{2u}-globulin was detected within the hepatocytes around the central vein (Fig. 5*A*) in a pattern consistent with previous observations (12). In rat–mouse chimeric livers, α_{2u}-globulin was detected in rat-derived areas (Fig. 5*B*) in a pattern similar to that of normal rat liver (Fig. 5*A*). While no α_{2u}-globulin was detected in control mouse kidneys (Fig. 5*C*), it was detected in the renal tubular epithelial cells of a transgenic mouse transplanted with rat liver cells (Fig. 5*D*).

DISCUSSION

To extend the usefulness of the Alb-uPA transgenic mouse model for studying liver cell growth, we introduced the Alb-uPA transgene into immunotolerant *nu/nu* mice. Transplantation of rat liver cells into these mice resulted in the complete reconstitution of mouse liver with rat hepatocytes. This is a remarkable demonstration that a functional liver can be formed from transplanted xenogeneic hepatocytes and suggests that Alb-uPA mouse livers can be reconstituted with hepatocytes from a range of species.

Transplanted rat hepatocytes responded to the Alb-uPA mouse liver environment and divided, replacing the transgenic parenchyma. Rat hepatocytes also responded to mouse modulatory influences, since the rat–mouse chimeric livers were of similar size to mouse control livers. These observations suggest that rat hepatocytes produced surface proteins that could interact appropriately with soluble mouse factors, with extracellular matrix, and with surface proteins on other mouse liver cells.

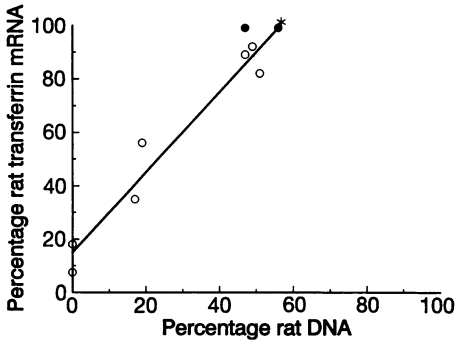

FIG. 4. Rat gene expression versus rat DNA in regenerated liver. Samples of regenerated liver from hemizygous (○) and homozygous (●) transgenic *nu/nu* mice transplanted with rat liver cells were analyzed for rat and mouse transferrin mRNA and for rat DNA. The percentage of rat transferrin mRNA was plotted as a function of rat DNA detected in the same sample. There was a linear correlation between the proportion of rat transferrin mRNA and rat DNA detected ($r = 0.9747$). The line ($y = 1.55x + 14.90$) represents the best fit as determined by regression analysis. Note that as the level of transferrin approaches 100%, the proportion of rat DNA approaches 60%, the estimated percentage of hepatocytes in mouse liver. *, The DNA of a third homozygous mouse liver was too degraded for analysis; mRNA from the same liver showed that 100% of the transferrin mRNA was rat derived (solution hybridization does not require full-length message).

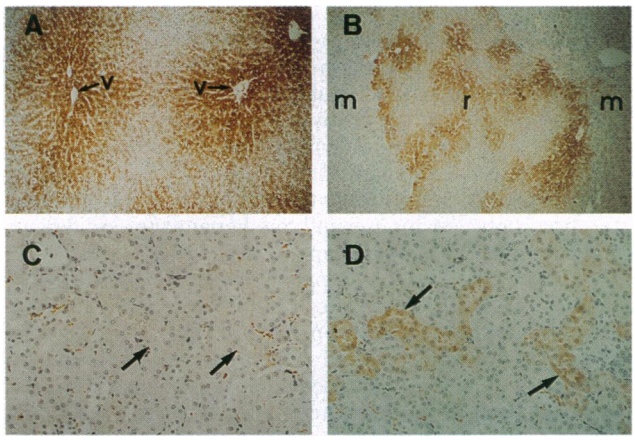

FIG. 5. Immunohistochemical detection of rat α_{2u}-globulin protein expression in transgenic mice. Formalin-fixed, paraffin-embedded sections of liver or kidney were immunostained with a polyclonal antiserum against rat α_{2u}-globulin and counterstained with hematoxylin. (*A*) Control rat liver. Central veins (v) are designated. (*B*) Liver of hemizygous transgenic *nu/nu* mouse transplanted with rat liver cells. Shown is a rat-derived area (r) in between two mouse-derived areas (m). In the rat-derived area, note the perivenous distribution of α_{2u}-globulin protein expression. This distribution is similar to that of the control rat liver shown in *A*. (*C*) Control mouse kidney. Renal tubules are indicated with arrows. (*D*) Kidney of hemizygous transgenic *nu/nu* mouse shown in *B*. Renal tubules are indicated with arrows. (*A* and *B*, ×40; *C* and *D*, ×75.)

Liver function was normal in both hemizygous and homozygous transgenic mice transplanted with rat liver cells. In some hemizygous animals, analysis of transferrin transcripts suggested that the majority of liver function was supplied by mouse hepatocytes. In homozygous animals, on the other hand, over 90% of hepatocellular gene expression was rat derived. Rat hepatocytes must therefore be supplying the liver function of these animals. Since total serum protein and serum albumin levels in animals with rat–mouse chimeric livers were similar to levels in mouse controls, it follows that synthesis of serum proteins by rat cells was appropriately regulated. Finally, the clinical health of the animals suggested that rat hepatocytes were performing other important hepatocellular functions, such as intermediate metabolism and detoxification of organic wastes, at a level appropriate to the mouse, and that the secreted rat hepatocellular proteins were functional in the mouse.

Transplanted rat hepatocytes appeared to grow between the existing portal tracts of the recipient livers, just as did transplanted mouse liver cells in previous transplantation experiments (1). In regenerating livers, portal triads were found not within nodules of donor-derived hepatocytes but on the periphery of the regenerating nodules, in host liver areas. In fully regenerated livers, portal tracts appeared to contain vestiges of host-derived parenchyma (Fig. 3*F*), suggesting that these portal tracts were those of the recipient liver. Further support that portal tracts were derived from the recipient mouse comes from the observation that the biliary ducts, a component of portal tracts, did not react with a rat-specific bile duct antibody (data not shown). It follows that other portal tract components, including vessels and associated connective tissue cells, are also most likely of mouse derivation. This is consistent with the observation that only 56% of DNA was rat-derived in livers showing nearly 100% rat hepatocellular gene expression. Presumably the mouse-derived DNA comes from nonhepatocytes including endothelial cells, biliary epithelial cells, and fibroblasts. Thus we have derived livers in which hepatocytes are derived from one species, the rat, and biliary epithelial cells

and presumably other accessory cells are derived from a second species, the mouse.

The generation of mice with livers composed of rat hepatocytes provides an approach for understanding differences in the biology of rat and mouse liver. For example, after partial hepatectomy, peak mitotic activity of hepatocytes is delayed by 24 hr in mouse liver compared to rat liver (15). It is unclear whether this difference in growth response is due to intrinsic differences between mouse and rat hepatocytes or due to differences in the liver environments in the two species. If the growth response to partial hepatectomy of mouse livers reconstituted with rat hepatocytes resembles that of rat livers, this would suggest that rat hepatocytes have some intrinsic differences from mouse hepatocytes that persist even in a mouse environment. Finally, the demonstration that Alb-uPA mouse livers can be reconstituted with rat hepatocytes raises the exciting possibility that they also can be reconstituted with human liver cells. These human–mouse livers could potentially be used as a repository for human hepatocytes, as reagents for human carcinogenicity studies, or as models for human liver disease.

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