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Integration of transplanted hepatocytes into host liver plates demonstrated with dipeptidyl peptidase IV-deficient rats

(liver cell plate/bile canaliculus/gap junction/acute liver failure/gene therapy)

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ABSTRACT **To analyze mechanisms of liver repopulation, we transplanted normal hepatocytes into syngeneic rats deficient in dipeptidyl peptidase IV activity. When isolated hepatocytes were injected into splenic pulp, cells promptly migrated into hepatic sinusoids. To examine whether transplanted hepatocytes entered liver plates and integrated with host hepatocytes, we analyzed sharing of hepatocyte-specific gap junctions and bile canaliculi. Colocalization studies showed gap junctions uniting adjacent transplanted and host hepatocytes in liver plates. Visualization of bile canalicular domains in transplanted and host hepatocytes with dipeptidyl peptidase IV and ATPase activities, respectively, demonstrated hybrid bile canaliculi, which excreted a fluorescent conjugated bile acid analogue. These results indicate that transplanted hepatocytes swiftly overcome mechanical barriers in hepatic sinusoids to enter liver plates and join host cells. Integration into liver parenchyma should physiologically regulate the function or disposition of transplanted hepatocytes and benefit applications such as gene therapy.**

Liver repopulation has significant potential for *ex vivo* gene therapy (1). The potential of *ex vivo* gene therapy was recently demonstrated in familial hypercholesterolemia, although the fate of transplanted hepatocytes in the liver was undefined (2, 3). Hepatocyte transplantation to salvage an acutely injured liver has similarly gained interest, although optimal results will require massive liver repopulation (1). Cells can be safely delivered into hepatic sinusoids; however, as the portal vascular bed has limited capacity, augmentation of the transplanted hepatocyte mass may require either cell proliferation or repeated cell transplantation. Such strategies should be facilitated by integration of transplanted hepatocytes into liver plates. Indefinite persistence of cells in hepatic sinusoids may impede blood flow or limit repeated cell transplantation. Although portal hypertension induced by transplanting cells into hepatic sinusoids is short-lasting (4), whether microcirculatory alterations accompany normalization of portal pressures is unknown. Moreover, nonavailability of cell–cell contact or autocrine signals to hepatocytes in hepatic sinusoids could limit proliferation or other biological processes. On the other hand, integration of transplanted hepatocytes into liver plates should restore position-specific hepatic gene expression and lineage-specific cellular life history (5). However, despite recent localization of genetically marked transplanted hepatocytes in liver (6–8), whether cells integrated into liver plates was unknown.

Based upon indefinite persistence of transplanted hepatocytes in liver (7, 8), we predicted that cells should enter liver plates, although this would require circumvention of the sinusoidal endothelium and space of Disse. To test this hypothesis, systems were employed that used mutant Fischer 344 rats lacking dipeptidyl peptidase IV (DPPIV) activity (9), which serves also as a marker of bile canaliculi (10). Cell integrations could be demonstrated by formation of specific hybrid organelles, such as bile canaliculi or gap junctions (11, 12).

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MATERIALS AND METHODS

Animals. Donor Fischer 344 rats were commercially obtained (Harlan–Sprague–Dawley). Twelve syngeneic DPPIV$^-$ recipients, ≈200 g each, were provided by the Special Animals Core of the Liver Research Center at the Albert Einstein College of Medicine. Hepatocyte isolation by collagenase perfusion and injection of 2×10^7 cells into the splenic pulp of each DPPIV$^-$ rat were as described (4). Animal use was approved by the Animal Care and Use Committee at the Albert Einstein College of Medicine. During 8 months of studies, recipients were killed at intervals for tissue analysis.

Liver Nonparenchymal Epithelial Cells. FNRL (Fischer 344 neonatal rat liver) cells served as controls (13). FNRL cells respond to hepatic stimulator substance (14) and react with H-4 and OV-6 monoclonal antibodies, which recognize hepatocyte and oval-cell antigens, respectively (15). When transformed, FNRL cells produce tumors of hepatocyte, biliary, or other lineages (16). The FNRL cells should have produced DPPIV$^+$ bile canaliculi after differentiation into hepatocytes *in vivo*.

Synthesis and Use of a Fluorescent Conjugated Bile Acid Analogue. To test biliary function, a fluorescent compound, N^{α}-choloyl-N^{ε}-NBD-lysine (CNBG) was synthesized in the laboratory of Alan Hofmann by Claudio D. Schteingart and Huong-Tu Ton-Nu. Briefly, the method involved coupling of 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD) to N^{α}-choloyllysine (17). A derivative of the naturally conjugated bile salt choloylglycine, the compound has a fluorophore (NBD) coupled by a five-atom tether to the α-carbon of glycine. The compound should have a pK_a of 3.9, on the basis of the ionization behavior of choloylglycine (18), and enters hepatocytes by a Na^+-dependent mechanism (19), presumably involving the Ntcp transporter (20). The protonated form of CNBG was dissolved in phosphate-buffered saline, pH 7.4 (PBS). The compound was empirically infused i.v. into a hepatocyte recipient at 4 μmol/min per kg of body mass (6 μmol, or 3 mg, over 10 min), although the V_{max} for CNBG transport is unknown. To detect CNBG excretion in bile canaliculi and for other analysis, tissues were frozen in cold methylbutane and stored at −80°C. Fluorescence was examined under a fluorescein isothiocyanate filter in cryostat sections secured on the microscope stage and DPPIV activity was then colocalized in tissues *in situ*.

Histological Studies. Cryostat sections, 5 μm thick, were fixed for 10 min in acetone/chloroform, 1:1 (vol/vol), at 4°C and incubated with a DPPIV substrate to demonstrate enzymatic activity (21). Biliary ATPase activity was detected by incubating sections with ATP (22). To colocalize DPPIV and ATPase activities, cryostat sections were reacted first for DPPIV activity, washed with PBS, and immediately processed for ATPase activity. Glucose-6-phosphatase activity was detected in unfixed cryostat sections (23). The 32-kDa major

Abbreviations: Cx, connexin; DPPIV, dipeptidyl peptidase IV.

hepatic gap junction protein, connexin 32 (Cx32), was localized by an anti-rat antibody (7C6, αCx32) (24). For immunostaining, cryostat sections were blocked with sheep serum and incubated with αCx32 (1:100 dilution), and antibody binding was detected with a peroxidase-based system (BioGenex Laboratories, San Ramon, CA).

RESULTS

Demonstration of Liver Repopulation. Utilization of DPPIV$^-$ recipients allowed the use of strategies described elsewhere (9). Optimal localization of transplanted cells required that all donor hepatocytes were DPPIV$^+$, and recipients, DPPIV$^-$ (Fig. 1 *A* and *B*).

Although the organization of hepatic lobules is complex, hepatocytes are arranged in liver plates surrounded by sinusoids containing blood and littoral cells, while bile drains into canaliculi between adjacent hepatocytes. In view of the intimate anatomic relationship between adjacent hepatocytes in normal liver, we reasoned that if transplanted cells were to integrate into liver plates, this should lead to the appearance of hybrid or shared organelles, such as bile canaliculi and gap junctions. Cell translocations into liver plates could be demonstrated by delineation of sinusoidal and bile canalicular domains of host or transplanted hepatocytes. To demonstrate shared gap junctions in the intercellular domains of transplanted and host hepatocytes in liver cell plates, we immunostained tissue sections previously reacted for DPPIV activity. Dual studies to simultaneously distinguish between gap junctions and transplanted hepatocytes did not require additional manipulations. However, to demonstrate hybrid bile canaliculi in transplanted and host hepatocytes, we developed additional experimental strategies. Briefly, sequential reactions for DPPIV and ATPase activities visualized donor and host hepatocytes containing differently colored bile canaliculi (Fig. 1 *C* and *D*).

Entry of Transplanted Hepatocytes into Liver Plates. We injected cells into spleen to reproducibly and safely deposit them into hepatic sinusoids (4, 25). Intrasplenic hepatocyte transplantation may confer additional advantages, such as tolerance to heterologous antigens and superior cellular gene expression (26, 27). When hepatocytes were transplanted into DPPIV$^-$ recipients, transplanted cells could be unequivocally localized in the liver (Fig. 2). Initially, transplanted cells were situated in portal spaces but entered host liver plates in <1 week. Hepatocyte recipients were killed at intervals and survival of transplanted DPPIV$^+$ hepatocytes was demonstrated for up to the 8-month duration of the studies, in agreement with previous findings (7, 8).

Demonstration of Hybrid Gap Junctions. Hepatic gap junctions are recognized in thin sections as septilaminar linear structures formed by apposed membranes with an extracellular gap (12). Hepatic Cx32 is several times more abundant than Cx26, although they colocalize in gap junction plaques. Therefore, we used αCx32 antibody to visualize hepatic gap junctions (Fig. 3). Reaction of tissues with αCx32 visualized gap junctions in the expected domains of adjacent hepatocytes but not in biliary cells. This strategy provided an excellent demonstration of hybrid gap junctions uniting adjacent transplanted and host hepatocytes (Fig. 3).

Demonstration of Functionally Intact Hybrid Bile Canaliculi. Bile canaliculi are ≈0.75 μm in diameter and are composed of membranes from adjacent cells (28). The canaliculi exhibit contractile responses, a variety of ectoenzymes, and energy-dependent transport systems (11). These mechanisms allowed us to utilize differential expression of DPPIV and ATPase activities to demonstrate integration of host and transplanted hepatocytes (Fig. 4). We found hybrid bile canaliculi containing components of DPPIV$^+$ donor and ATPase$^+$

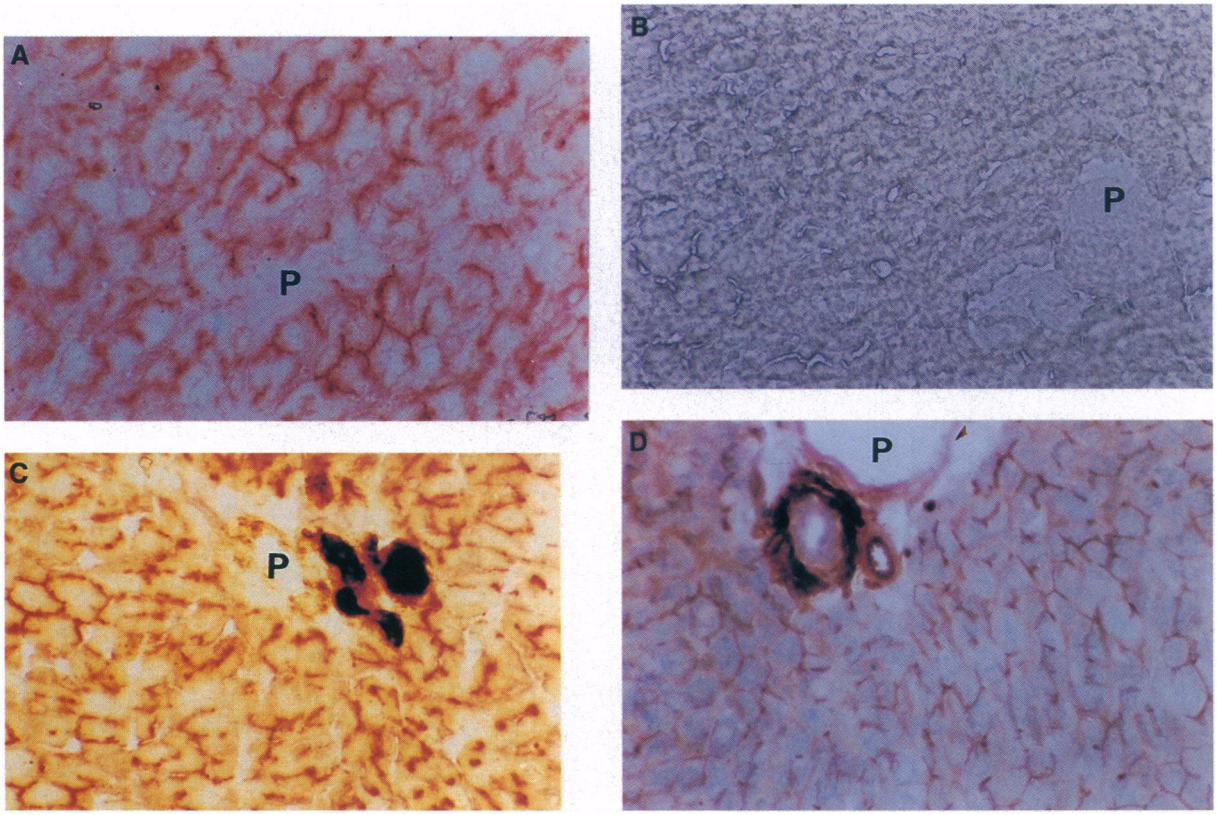

FIG. 1. (*A*) DPPIV staining of a normal F344 donor liver. All hepatocytes but not bile ducts are stained (P, portal area). (*B*) DPPIV$^-$ rat liver showing absence of DPPIV activity. (*C* and *D*) Sequential staining for DPPIV, followed by ATPase activity. (*C*) Donor liver shows red DPPIV$^+$ bile canaliculi, whereas bile ducts exhibit ATPase activity (in brown). (*D*) DPPIV$^-$ liver shows only ATPase activity in bile canaliculi. (×170.)

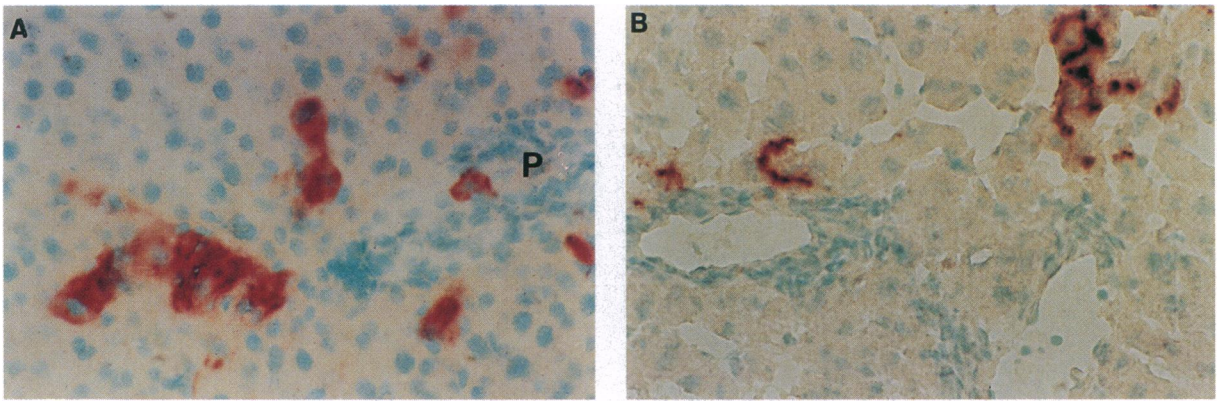

FIG. 2. (*A*) DPPIV$^+$ cells in vascular spaces 2 hr after transplantation. (*B*) Transplanted cells in liver plates after 7 days show well-defined bile canaliculi. (Methyl green counterstain; ×340.)

host hepatocytes in liver plates. Hybrid bile canaliculi could have developed only when transplanted hepatocytes physically joined host hepatocytes in liver plates. Use of hybrid bile canaliculi as an indicator showed that transplanted hepatocytes were fully integrated in liver plates within 7 days after transplantation, with indefinite survival in hosts up to the 8-month duration of the experiment. In contrast, when FNRL cells were transplanted into DPPIV$^-$ recipients, no bile canaliculi formed and DPPIV staining was negative (data not shown), which suggested absence of differentiation into hepatocytes.

Transplanted hepatocytes retained glucose-6-phosphatase activity within host liver plates, indicating preservation of hepatic gene expression (data not shown). To demonstrate excretory function in hybrid bile canaliculi, we used a fluorescent bile salt analogue. When the fluorescent bile salt was administered several weeks after transplantation of syngeneic DPPIV$^+$ hepatocytes, excretion of the infused bile salt was apparent in bile canaliculi throughout the liver, including hybrid bile canaliculi formed by transplanted cells (Fig. 5).

DISCUSSION

This study provides unequivocal evidence for entry of transplanted hepatocytes into liver plates and integration with host hepatocytes. The ability of transplanted hepatocytes to traverse the sinusoidal endothelium and space of Disse is remarkable. Direct access to the space of Disse is regulated by endothelial cell fenestrae ≈150 nm in diameter, although this dimension depends upon the contractile state of the perifenestral cytoskeleton (11). Delineation of the precise mechanisms will require further studies; however, differences in the size of hepatocytes (20–35 μm) and fenestral openings indicate that

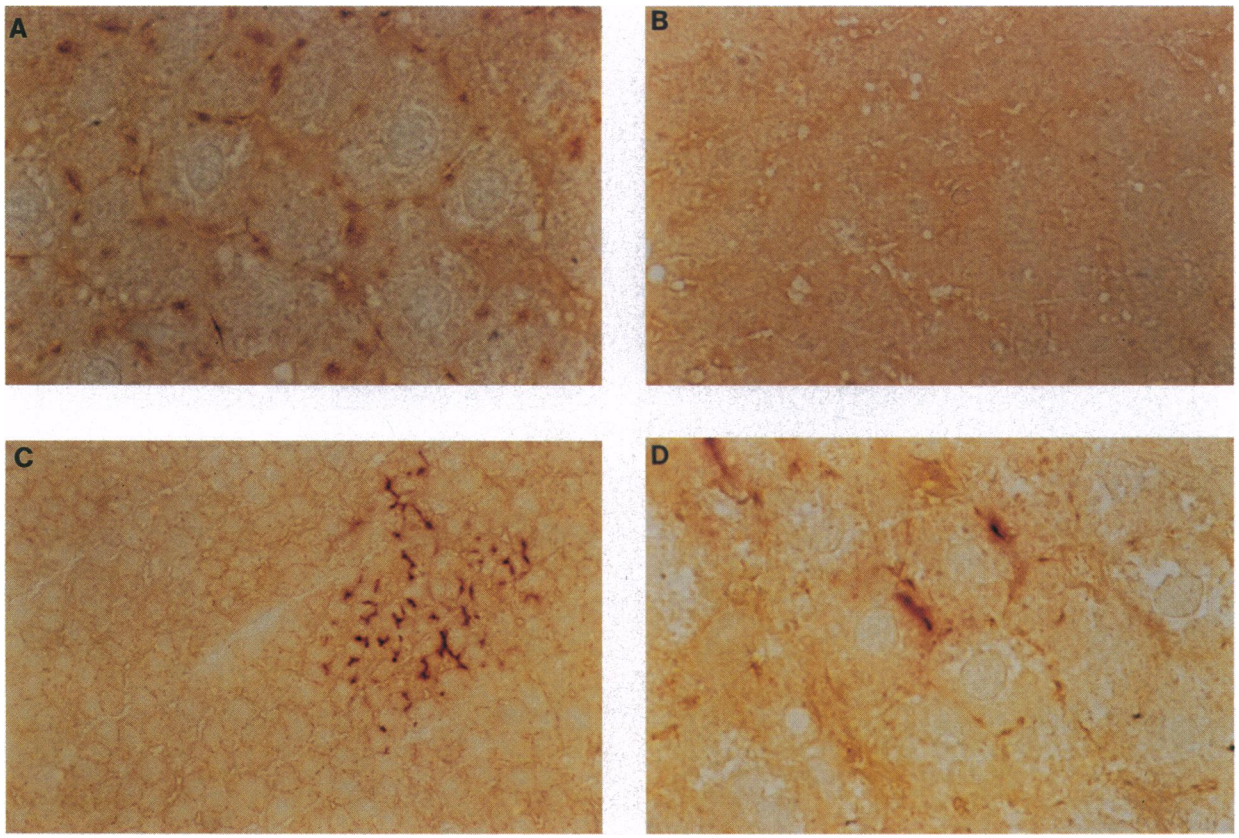

FIG. 3. (*A*) Normal rat liver showing circumscribed gap junctions in intercellular hepatocyte domains. (*B*) Negative control with omission of αCx32 antibody. (*C*) Recipient liver showing gap junctions in a group of transplanted cells. (*D*) Higher-power view of gap junctions joining a transplanted hepatocyte (red bile canaliculi) and host hepatocytes. (*A*, *B*, and *D*, ×850; *C*, ×170.)

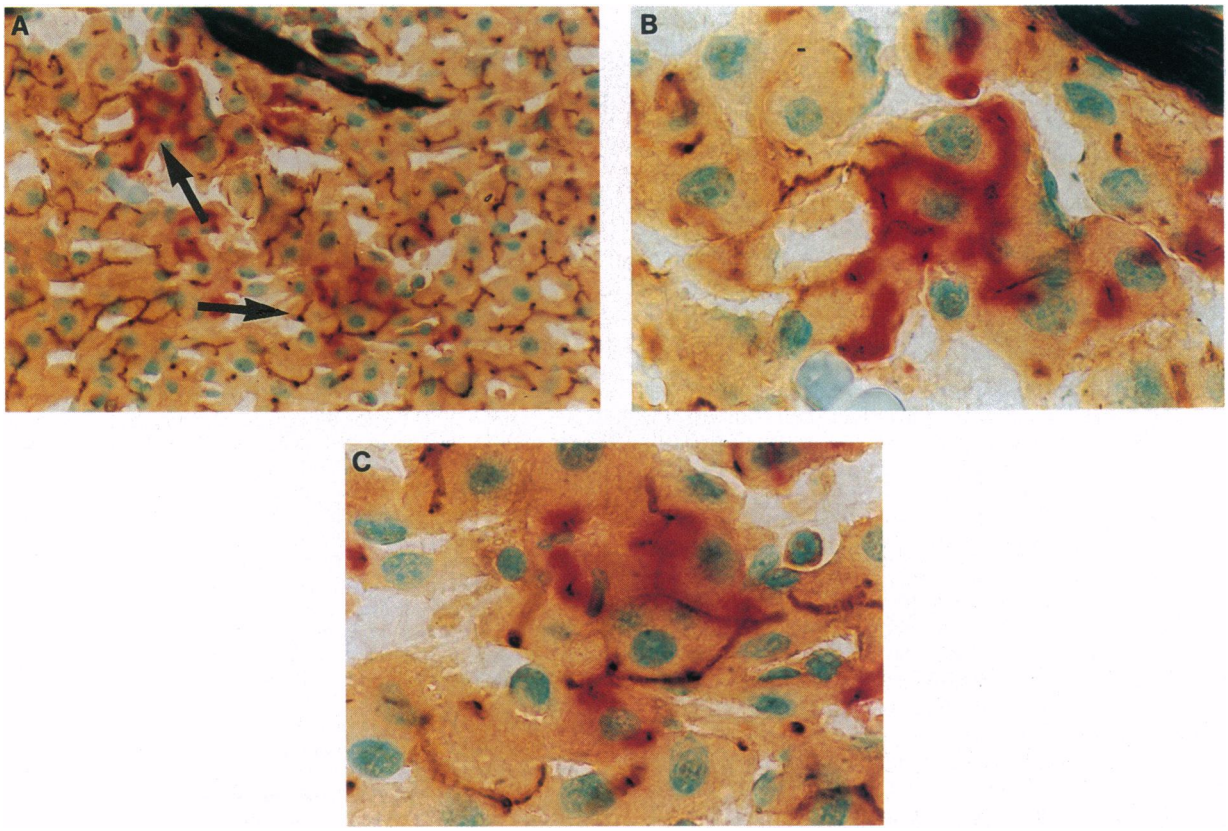

FIG. 4. (*A*) Hybrid bile canaliculi in hepatocyte recipients after DPPIV and ATPase reactions. Bile canalicular components in transplanted cells are red. Note ATPase activity in bile canaliculi, as well as bile ducts seen in top center. Higher-magnification views are shown (*B* and *C*) of areas marked with arrows. (×170.) (*B* and *C*) Interconnected networks of bile canaliculi draining host and transplanted hepatocytes. In some areas, brown and red colors overlap, indicating contributions from transplanted and host cells. Elsewhere, bile canalicular networks extend from transplanted cells into host hepatocytes. (×850.)

remodeling must occur during entry of transplanted cells into liver plates.

Maintenance of polarity in differentiated epithelial cells is critical for specialized functions. Restoration of normal hepatocyte polarity—as defined by the sinusoidal domain abutting the space of Disse, the intercellular domain containing junctional complexes with interspersed gap junction channels, and the apical or canalicular domain between adjacent cells—required integration into liver plates and fusion of cell membrane organelles between juxtaposed transplanted and host cells. The gap junction channels may occupy ≈3% of the hepatocyte cell membrane with 12 identical subunits, 6 of which, termed a connexon or hemichannel, are contributed by each cell (12). We hypothesized that hybrid gap junctions would require connexons from transplanted and adjacent host hepatocytes. Light microscopy was adequate for visualizing gap junctions in hepatocytes because, although individual channels may measure only 15–18 nm, gap junctional plaques could exceed 1 μm in diameter (12). Expression of gap junction proteins in the liver is cell type specific. Bile duct cells and littoral cells express Cx43 (29), whereas hepatocytes express Cx32 and/or Cx26 as major gap junction proteins (30). In the rat liver, the Cx32 gap junction protein is ≈10-fold more abundant than Cx26. Also, Cx32 protein is uniformly expressed

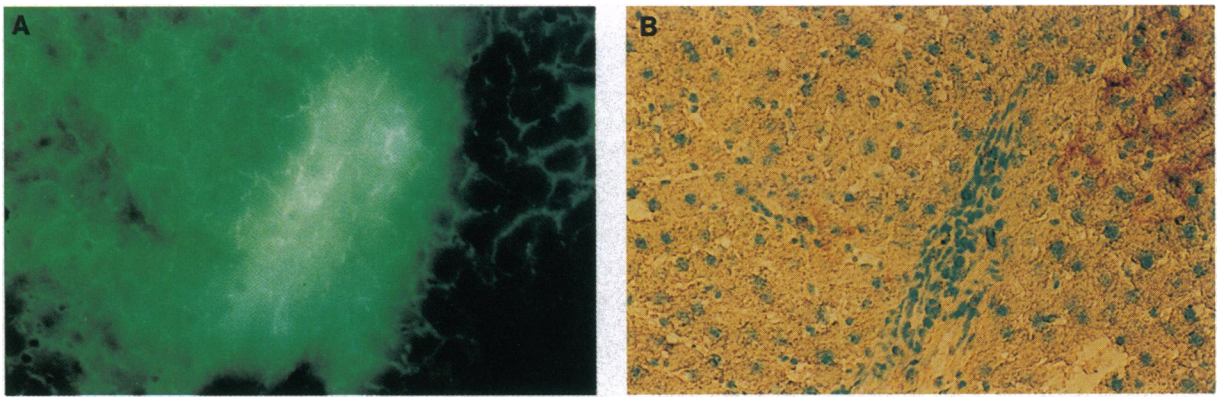

FIG. 5. (*A*) Fluorescence microscopy of liver from a hepatocyte recipient after infusion of a fluorescent bile acid analogue (see *Materials and Methods*). A bright fluorescent portal area surrounded with a lattice of bile canaliculi is visible after excretion of the bile acid analogue. (*B*) Same area of the liver as in *A* after *in situ* fixation with acetone/chloroform and reaction for DPPIV activity. Transplanted hepatocytes are present in upper left corner and elsewhere.

throughout the hepatic lobule, whereas Cx26 protein is preferentially expressed in periportal hepatocytes with a gradient across the hepatic lobule, which could potentially be helpful for examining cellular differentiation in transplanted cells.

Formation of hybrid bile canaliculi in transplanted hepatocytes but not in nonhepatocytes indicates that this feature could also be used as a parameter of cell differentiation. Despite dissociation with collagenase, isolated hepatocytes retain DPPIV and ATPase activities, although in a diffuse pattern consistent with disruption of bile canaliculi. Hepatocytes in the intact liver also maintain DPPIV and ATPase activities despite perturbations such as partial hepatectomy or carbon tetrachloride-induced liver injury (ref. 31, and S.G., unpublished observations). Therefore, our strategy to colocalize bile canaliculi in transplanted and host hepatocytes was effective and proved to be more convenient than cumbersome alternatives using electron microscopy.

Integration of transplanted hepatocytes in the liver parenchyma is particularly significant for massive liver repopulation. Until recently, the magnitude of liver repopulation by hepatocyte transplantation has been limited to 1–2% of the host liver (7, 8). However, in studies to be reported elsewhere, we have been able in one session to transplant hepatocytes representing up to 10–15% of the hepatic mass. Rapid translocation of transplanted hepatocytes from the sinusoids into the liver cell plate should facilitate testing additional strategies for massive liver repopulation, such as by repeated cell transplantation. Integration of transplanted hepatocytes in liver plates will allow studies to define their proliferative potential in the hepatic lobule, which could represent an alternative strategy for massive liver repopulation. Transplanted hepatocytes express cellular genes more effectively in the liver than in ectopic sites such as spleen, peritoneal cavity, and dorsal fat pad (27). Although regulation of hepatic gene expression is complex, with contributions from extracellular matrix components, cell–cell interactions, exposure to specific hormones or growth factors, and position in the hepatic lobule (5), integration of transplanted cells into the liver plate should maximize the potential for cellular gene expression and proliferation.

Our systems described here will be particularly relevant for studies of differentiation in hepatic progenitor cells. Although evidence for activation of progenitor liver cells has been provided, suitable bioassays are necessary to analyze the differentiation and fate of these progenitors *in vivo*. Development of hybrid bile canaliculi appears to be specific for hepatocytes, since we were unable to demonstrate hybrid bile canaliculi when FNRL cells were transplanted, presumably due to lack of their differentiation into hepatocytes. One reason for interest in progenitor cells would be their capacity for greater proliferation, which should be facilitated by integration of hepatocytes into liver parenchyma. Gap junction channels could contribute to cell proliferation by facilitating cellular signal transduction. Incorporation of transplanted cells into the liver plate will facilitate analysis of whether hepatocytes stream from portal toward pericentral areas, which will improve an understanding of their natural life history. Finally, unencumbered entry and assimilation of hepatocytes into liver cell plates suggest that particle size may only partly dictate fenestral regulation of trafficking into the space of Disse, specially when biological agents are involved. Our findings should also be relevant for workers investigating *in vivo* gene therapy.

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