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Retrovirus-mediated transduction of adult hepatocytes

(gene transfer/primary liver culture/hepatocyte-specific cytochemistry)

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ABSTRACT **Retrovirus-mediated gene transfer was used to develop a method for introducing genes into primary cultures of adult rat hepatocytes. Subconfluent monolayers of hepatocytes, cultured in hormonally defined media on different matrix substrata, were infected with helper-free stocks of a replication-defective retrovirus that constitutively expresses high levels of β-galactosidase. Retrovirus-mediated transduction was measured by two methods: (*i*) an *in situ* cytochemical stain that specifically detects the expression of viral expressed β-galactosidase, and (*ii*) Southern blot analysis, which measures the relative copy number of integrated provirus. Maximal transduction efficiency of ≈25% was achieved when the cells were infected after 3 days in culture; matrix had little effect on transduction efficiency. Enzyme cytochemical (catalase and glucose 6-phosphatase) and peroxidase immunocytochemical (asialoglycoprotein and UDP-glucuronosyltransferase) analyses of the cultures indicated that >95% of cells were hepatocytes. The demonstration of hepatocyte-specific organelles in cells expressing the viral-directed β-galactosidase provided unambiguous evidence for the transduction of hepatocytes. These methods should be useful in the development of liver-directed somatic gene therapy and in the study of liver-specific gene regulation.**

The liver is a metabolically rich organ that is the subject of studies involving many aspects of molecular biology including carcinogenesis, regeneration, and developmental and tissue-specific gene regulation. Hepatoma cell lines derived from spontaneous or induced tumors have been used to study some of these processes *in vitro*. However, the suitability of hepatoma cell lines for studying normal biological processes is questionable since they are malignantly transformed and the specific type of cell from which they were derived is often difficult to identify (1–3). An alternative *in vitro* system that has been developed and exploited by many investigators is the primary culture of adult rat hepatocytes.

Several methods have been described for isolating highly enriched populations of adult rat hepatocytes and maintaining these cells in culture for extended periods of time (4–7). Under optimal conditions, the cells undergo one or two rounds of division but cannot be passaged. In addition, metabolic functions unique to the hepatocyte can be maintained in culture for variable periods of time (e.g., transcription of liver-specific genes and detoxification of carcinogens).

One limitation of hepatocyte cultures for studying the molecular aspects of processes such as gene regulation is the lack of efficient gene transfer techniques. Conventional methods of transfection are inefficient and toxic to the cells (8). We have used recombinant retroviruses to overcome similar problems in several other systems including secondary cultures of fibroblasts (9) and keratinocytes (10) and primary cultures of bone marrow (11). In this report, we describe the efficient and stable transduction of primary cultures of adult rat hepatocytes by replication-defective retroviruses.

METHODS

Hepatocyte Isolation and Culture. Rat hepatocytes were prepared by the procedure of Berry and Friend (12) with the perfusion mixture of Leffert *et al.* (13). Male Sprague–Dawley rats (200–250 g) were used as the source of hepatocytes. Cells were plated at a density of 4×10^4 cells per cm^2 onto one of several matrix substrata in hormonally defined medium (7) supplemented with 10% fetal bovine serum. Four hours later, the medium was replaced with fresh hormonally defined medium, which was changed every 24 hr for the duration of the experiment. The following substrata were used. (*i*) Tissue culture plastic: Primaria plates from Falcon were used without additional preparation. (*ii*) Type I collagen: 10-cm tissue culture dishes were coated with type I collagen prepared from rat tail tendons (14). Briefly, collagen was solubilized in 0.1% acetic acid (3 mg/ml) and applied to plates (1 ml per 10-cm plate), which were exposed to NH_3 vapors, air-dried, sterilized by γ irradiation (10,000 rads), and hydrated with medium. (*iii*) Laminin: purified laminin from Collaborative Research was applied to tissue culture plates according to the recommendations of the manufacturer. (*iv*) Type IV collagen: 10-cm dishes coated with purified type IV collagen were kindly provided by L. M. Reid (Albert Einstein College of Medicine).

Virus Preparation and Hepatocyte Infection. A helper-free amphotropic producer of the BAG virus was provided by C. Cepko (Harvard University). The retroviral vector used to make this producer has been described (15); its structure is shown in Fig. 1. This virus coexpresses β-galactosidase from *E. coli* and the bacterial gene that confers resistance to neomycin in prokaryotes and to G418 in eukaryotes (*neo*). The producer was maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Unconcentrated viral stocks were prepared and titered as described (16); titers ranged from 1 to 4×10^5 colony-forming units per ml when tested on NIH 3T3 cells. Hepatocyte cultures were infected for 12 hr with viral stocks (5 ml of viral stock per 10-cm plate of hepatocytes) containing 8 μg of Polybrene per ml.

Southern Blot Analysis. High molecular weight cellular DNA was isolated as described (17) and aliquots (7.5 μg) were digested with either *Kpn* I or *Hin*dIII. The restriction fragments were resolved by electrophoresis in 1% agarose gels and analyzed according to the method of Southern by standard procedures (17). The blot was probed with the *Bam-*

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Abbreviations: *neo*, gene from bacteria that confers resistance to neomycin in prokaryotes and to G418 in eukaryotes; LTR, long terminal repeat sequences.

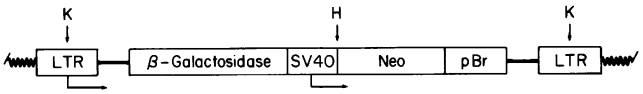

FIG. 1. Structure of the BAG vector. Arrows below vector indicate sites of transcription initiation, while wavy lines indicate flanking sequences. SV40, promoter from simian virus 40; Neo, *neo* gene; pBr, origin of replication in pBR322; K, *Kpn* I site; H, *Hin*dIII site.

HI/*Hin*dIII fragment of the neomycin gene that was labeled to high specific activity with [^{32}P]dCTP by the random primer method (18).

Cytochemical and Immunocytochemical Procedures. Cells infected with the BAG virus constitutively produce high levels of cytoplasmic β-galactosidase (15). Activity of β-galactosidase was detected *in situ* with the substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside, which forms a blue precipitate in infected cells (15). Immunocytochemical localization of UDP-glucuronosyltransferase and asialoglycoprotein receptor was performed in the culture dishes with horseradish peroxidase conjugated to protein A (from *Staphylococcus aureus*) and diaminobenzidine cytochemistry at pH 7.4 to detect peroxidase activity (19). Monospecific IgG to rat UDP-glucuronosyltransferase was purified from rabbit antiserum (20). R. Stockert (Albert Einstein College of Medicine) kindly provided monospecific antibody to rat asialoglycoprotein receptor. Controls for the immunocytochemical experiments included exposure of cells to preimmune rabbit sera followed by procedures identical to those used for specific rabbit antibody. Glucose 6-phosphatase activity was detected by the lead phosphate enzyme cytochemical procedure (21). Peroxisomes were visualized by demonstrating catalase activity with diaminobenzidine cytochemistry at pH 9.7 (22). Cultures analyzed for both peroxisomes and β-galactosidase activity were first assayed *in situ* for β-galactosidase activity as described above followed by immediate analysis for catalase activity.

RESULTS

Hepatocyte Infection. Hepatocytes were isolated from adult rats, plated at subconfluent densities on matrix substrata, and maintained in the hormonally defined medium described by Enat *et al.* (7). The cultures were infected with an amphotropic retrovirus derived from a previously described retroviral vector called BAG (15); the structure of this vector is shown in Fig. 1. The transcript initiated at the 5′ long terminal repeat sequences (LTR) of this virus is responsible for expression of β-galactosidase, while that initiated at the simian virus 40 promoter results in expression of the *neo* gene. Hepatocytes were analyzed for retroviral-mediated transduction 36–48 hr after the infection was initiated. Transduction efficiency was optimized with respect to the time of exposure to virus and the matrix substrata on which the hepatocytes were plated.

The efficiency of transduction was initially assessed by directly measuring the integration of provirus. High molecular weight DNA from transduced cultures of hepatocytes was digested with *Kpn* I and analyzed by the method of Southern with a probe that is complementary to sequences unique to the provirus (i.e., the *neo* gene). *Kpn* I has recognition sites in the LTR sequences; consequently, each integrated provirus will be contained in a 6.9-kilobase restriction fragment irrespective of the site of integration (Fig. 1). The intensity of the resulting band on the autoradiograph is proportional to the number of proviral integrants in the population. Fig. 2*A* presents a Southern blot of hepatocytes cultured on one of several forms of matrix substrata (type I collagen, laminin, type IV collagen, and tissue culture plastic) and infected on day 1, 2, 3, 4, or 5. Hepatocytes on each matrix substrata exhibited a consistent pattern of susceptibility to transduction; proviral integration increased from virtually undetectable on day 1 to maximal on day 2 or 3, and subsequently diminished to low levels by day 5. Maximal proviral integration, which was essentially independent of matrix, occurred when cultures were infected on day 2 for cells on tissue culture plastic, or day 3 for cells on type I collagen, laminin, or type IV collagen.

Further experiments were performed to show that the viral DNA detected in Fig. 2 was actually integrated into hepatocyte DNA as opposed to existing as an extrachromosomal form of linear or circular DNA. The same DNAs were digested with *Hin*dIII (a restriction enzyme with a single site in the proviral DNA) and subjected to Southern blot analysis with a *neo* probe. If the viral DNA exists as an integrated provirus, no distinct *Hin*dIII fragments will be detected because the outer borders of these fragments are located in flanking DNA and therefore are heterogeneous. In fact, no predominant *Hin*dIII fragments were visualized when this analysis was done (data not shown), suggesting that the majority of viral DNA is integrated into genomic DNA.

NIH 3T3 cells were infected with the same viral stocks used to infect hepatocytes (Fig. 2*A*). Southern blot analysis demonstrated little variation in the titer of the viral stocks; the estimated proviral copy number ranged from 0.5 to 0.7 copy per cell. This estimate of copy number was based on a comparison to samples with known quantities of standard

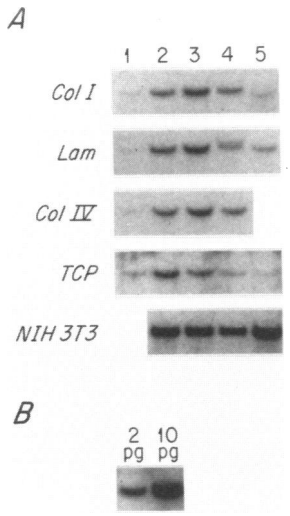

FIG. 2. Effect of extracellular matrix and time of infection on integration of provirus in hepatocyte cultures. (*A*) Adult rat hepatocytes isolated from a single collagenase perfusion were plated on 10-cm plates coated with one of several forms of matrix substrata. Single plates of hepatocytes from each category of matrix were infected with fresh preparations of viral stocks and analyzed for copy number of integrated provirus 36–48 hr after the infection was initiated (see *Methods* for details). A 3-day exposure of a Southern blot is shown. A single band was visualized in each lane; the area of the autoradiograph containing this band is shown. Lanes 1–5 indicate the days the cells were infected. Top four series of bands represent hepatocytes cultured on different forms of matrix: Col I, type I collagen; Lam, laminin; Col IV, type IV collagen; TCP, tissue culture plastic. Bottom series of bands shows an identical analysis of NIH 3T3 cells infected with the same viral stocks used to infect hepatocyte cultures. (*B*) Various amounts (2 and 10 pg) of the purified BAG plasmid were mixed with 7.5 μg of uninfected NIH 3T3 DNA and analyzed as described in *A*. A single band was detected that comigrated with the bands shown in *A*. Data from *A* and *B* were derived from a 3-day exposure of the same Southern blot. We estimate that 2 and 10 pg of plasmid in 7.5 μg of NIH 3T3 DNA correlates to approximately 0.3 and 1.2 copies of provirus per cell, respectively (see *Results* for details).

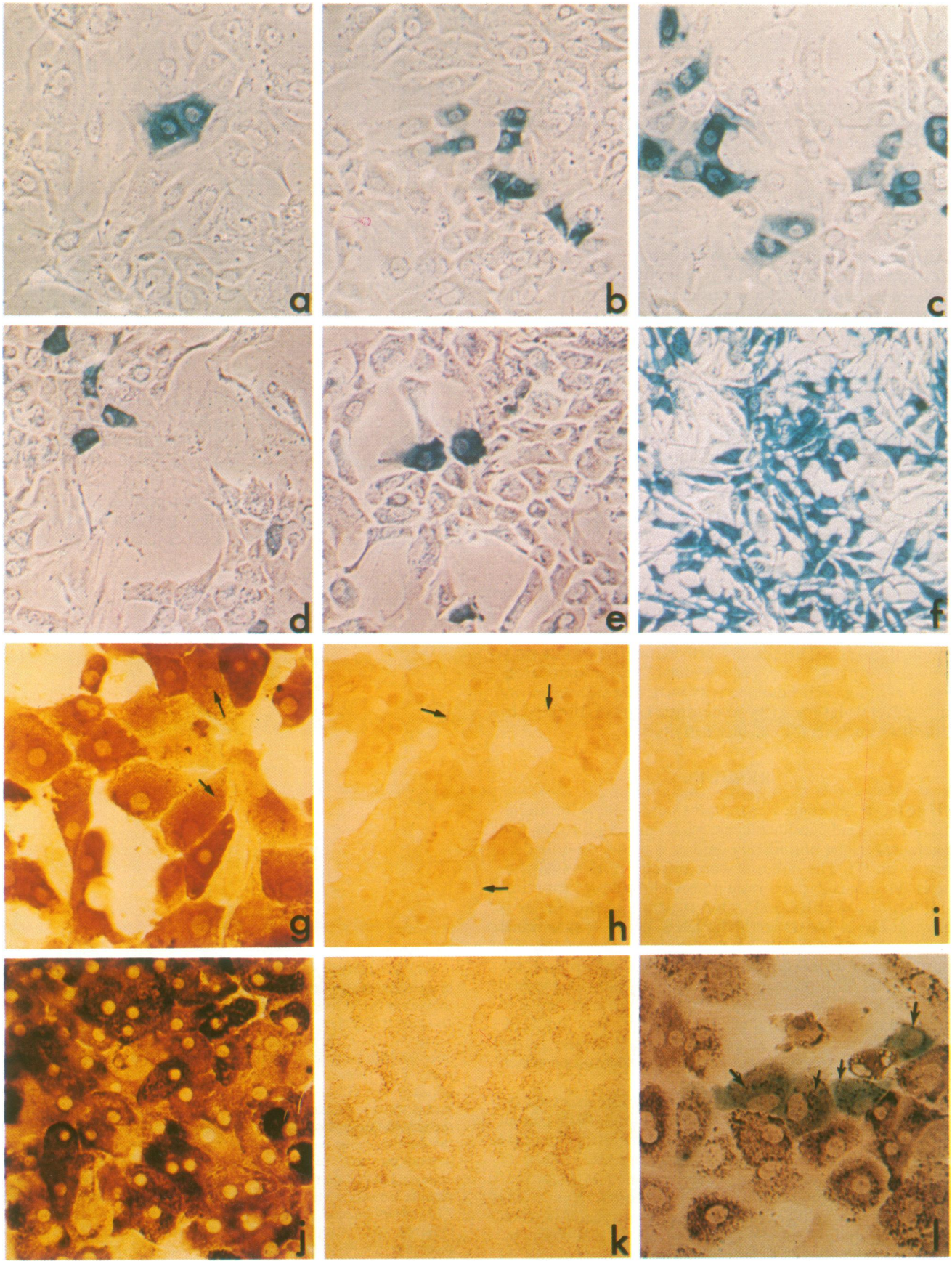

FIG. 3. (*a–f*) Cytochemical localization of β-galactosidase activity in transduced cultures of hepatocytes and NIH 3T3 cells. Duplicate plates of the experiment described in the legend to Fig. 2 were stained for the expression of β-galactosidase as described in *Methods*. (*a–e*) Hepatocyte cultures plated on type I collagen and infected on days 1–5, respectively. (*f*) A population of NIH 3T3 cells was infected with the same viral stock used to infect hepatocytes on day 3. (*g–k*) Liver-specific cytochemical and immunocytochemical stains of rat hepatocyte cultures.

plasmid (Fig. 2*B*) along with the assumption that NIH 3T3 cells are hypotetraploid (i.e., approximately 3N). The estimated copy number of proviral integrants in maximally infected hepatocytes (e.g., Fig. 2 Lam, lane 3) is ≈0.2 copy per cell, assuming that the DNA content of NIH 3T3 cells is equal to that of hepatocytes. This assumption is probably valid since the majority of hepatocytes in culture are either tetraploid or octaploid (23).

Duplicate cultures of infected hepatocytes were analyzed *in situ* for retrovirus transduction (and expression) by the cytochemical stain for β-galactosidase (15). This procedure specifically labels cells that express viral-directed β-galactosidase; endogenous β-galactosidase is not detected (data not shown). Fig. 3 (*a–e*) shows an analysis of hepatocytes plated on type I collagen and infected on day 1, 2, 3, 4, or 5. The efficiency of transduction, as measured cytochemically, exhibited the same dependence on time in culture as was demonstrated by Southern blot analysis. The fraction of labeled cells increased from <1% in cultures infected on day 1 to ≈25% when infected on day 3; the transduction efficiency dropped dramatically in cultures infected on the next 2 days (days 4 and 5).

Similar analysis of NIH 3T3 cells infected with the same viral stock used to infect day 3 hepatocytes indicated that ≈50% of the cells were labeled (Fig. 3*f*). This is consistent with the estimated efficiency of transduction based on Southern blot analysis (Fig. 2*A*).

Cytochemical Characterization of Hepatocyte Cultures. We used a series of liver-specific cytochemical and immunocytochemical stains to rigorously document the cellular composition of the hepatocyte cultures.

UDP-Glucuronosyltransferase. Chowdhury *et al.* (20) used immunocytochemical techniques to determine the distribution of UDP-glucuronosyltransferase in the liver. This membrane-bound enzyme is present exclusively in hepatocytes and is localized to the endoplasmic reticulum and nuclear membrane. Immunocytochemical analysis of 3-day-old hepatocyte cultures by using a monospecific polyclonal antibody to UDP-glucuronosyltransferase shows reaction product distributed in cytoplasmic clumps and at the periphery of the nucleus in >95% of the cells (Fig. 3*g*); these reactive sites correspond to the endoplasmic reticulum and the nuclear envelope, respectively. No reaction product is seen in experiments performed with preimmune rabbit IgG (Fig. 3*i*).

Asialoglycoprotein Receptor. This well-described receptor is specifically expressed in hepatocytes (24). Immunocytochemical analysis in rat liver localizes this receptor to a domain of the plasma membrane that borders the sinusoids; under light microscopy, the receptor is seen at the perimeter of the hepatocyte along its sinusoidal face (24, 25). The level of asialoglycoprotein receptor decreases in culture; however, it is still demonstrated in virtually all cells of a 3-day-old hepatocyte culture. Reaction product is seen as a dense line in focal regions of the hepatocyte periphery (Fig. 3*h*). This characteristic staining is absent in experiments with control rabbit serum (Fig. 3*i*).

Glucose 6-Phosphatase. This glycolytic enzyme is a well-recognized cytochemical marker for hepatocytes. It can be detected in virtually all hepatocytes of liver sections; however, there is marked regional variation in enzyme activity with the greatest activity found in the periportal region (26). We used a simple cytochemical stain for glucose 6-phosphatase in 3-day-old hepatocyte cultures. Characteristic brown/black cytoplasmic staining was seen in >95% of the cells (Fig. 3*j*). As expected, there was marked cell-to-cell variation in enzyme activity. Activity was still present at a slightly diminished level in >95% of cells after 5 days in culture (data not shown). No activity was detected in pure cultures of nonparenchymal cells such as fibroblasts or endothelial cells (data not shown).

Peroxisomes. We used the method of Novikoff *et al.* (22) to visualize the distribution of peroxisomes in hepatocyte cultures. These small cytoplasmic structures (diameter, ≈0.5 μm) are found specifically in hepatocytes (in the context of the liver) and are visualized by cytochemical staining for catalase (27). Again, >95% of the cells in 3-day-old cultures demonstrated numerous catalase-positive peroxisomes, appearing as dot-like structures distributed randomly throughout the cytoplasm (Fig. 3*k*). The same proportion of cells contained peroxisomes after 5 days in culture; however, the intensity of catalase staining was slightly diminished and the peroxisomes aggregated in an area surrounding the nucleus (data not shown). Peroxisomes were not detected when pure cultures of nonparenchymal cells (e.g., fibroblasts) were analyzed (data not shown).

To conclusively document transduction of hepatocytes, cultures were infected on day 3 and analyzed for the presence of both peroxisomes and viral-directed β-galactosidase 48 hr later (Fig. 3*l*). The β-galactosidase reaction was carried out for short time periods (4–8 hr in *l* versus 16–24 hr in *a–f*) because the blue product of the reaction partially obscures the catalase-stained peroxisomes. Peroxisomes were clearly detected in >95% of all cells expressing β-galactosidase. An example of this is presented in Fig. 3*l*.

DISCUSSION

Factors important for the efficient transduction of any cell with a retrovirus include the presence of the receptor for the viral envelope protein on the cell's plasma membrane as well as ongoing DNA synthesis. Adult hepatocytes demonstrate several features that make retrovirus-mediated transduction a potentially challenging problem. Adult hepatocytes in culture have limited regenerative potential (one or two divisions per cell) and cannot be passaged (4–7). In addition, primary cultures of hepatocytes contain various amounts of nonparenchymal cells, thereby complicating an accurate assessment of hepatocyte-specific transduction (4–7).

In our system, hepatocytes were isolated and cultured under conditions that minimized the presence of nonparenchymal cells and maximized the division of parenchymal cells (7). Four independent cytochemical and immunocytochemical analyses documented unambiguously the cellular composition of our cultures. At a time when the cultures were most susceptible to transduction (3 days), >95% of the cells exhibited enzyme activities (glucose 6-phosphatase, UDP-glucuronosyltransferase, and catalase), receptors (asialoglycoprotein), and subcellular organelles (peroxisomes) characteristic of hepatocytes in the intact liver.

Hepatocytes were isolated by collagenase perfusion, plated on type I collagen, and cultured in hormonally defined medium for 72 hr prior to analysis. (*g*) Immunocytochemical localization of UDP-glucuronosyltransferase by using a monospecific antibody. Arrows indicate presumed antigen localization at the periphery of the nucleus (nuclear envelope) and in cytoplasmic clumps (endoplasmic reticulum). (*h*) Immunocytochemical localization of asialoglycoprotein receptor with a monospecific rabbit antibody. Arrows indicate antigen localized to the plasma membrane. (*i*) Immunocytochemical analysis with preimmune rabbit serum. (*j*) Cytochemical localization of glucose 6-phosphatase activity. Reaction product is found in cytoplasmic clumps (endoplasmic reticulum) and in a rim encircling the nucleus (nuclear envelope). (*k*) Cytochemical localization of catalase in peroxisomes. (*l*) Cytochemical localization of peroxisomes and viral directed β-galactosidase in the same cells. Arrows indicate examples of peroxisomes. (*a–f*, ×170; *g–l*, ×250.)

Transduction of hepatocytes was documented by measuring the integration of proviruses in the population as well as labeling transduced cells *in situ* with a cytochemical stain that detects viral expression. The demonstration of hepatocyte-specific organelles (peroxisomes) in cells expressing a viral-directed gene (β-galactosidase) provided conclusive evidence for retrovirus-mediated transduction of adult hepatocytes. Transduction was maximal when the cells were infected after 3 days in culture. The relationship between time in culture and susceptibility to infection remains unexplained; however, it is intriguing that susceptibility to infection closely parallels the rate of DNA synthesis that has been measured previously in similar kinds of hepatocyte cultures (5). The virus used in our study was derived from an amphotropic packaging cell line and was of moderate titer (2×10^5 colony-forming units per ml when titered on NIH 3T3 cells). Attempts to infect rat hepatocytes with high-titer ecotropic virus were not as successful (J.M.W. and R.C.M., unpublished data). We have used simple modifications of the methods described in this paper to efficiently transduce hepatocytes from other species including mouse and rabbit (J.M.W. and R.C.M., unpublished data).

During the preparation of this manuscript, two groups reported the transduction of cultured hepatocytes with replication-defective retroviruses: Wolff *et al.* (28) described the transduction of adult rat hepatocytes with viruses that express the *neo* gene or the gene for hypoxanthine-guanine phosphoribosyltransferase, while Ledley *et al.* (29) reported the transduction of neonatal mouse hepatocytes with viruses that express the *neo* gene. The latter experiments were facilitated through the use of neonatal cells, which, in contrast to adult hepatocytes, undergo marked proliferation *in vitro*. Hepatocyte-specific transduction was documented in both studies by exposing the cultures to a virus that expresses the *neo* gene, placing the transduced cultures into medium that selects for cells expressing the *neo* gene, and detecting albumin secretion or phenylalanine hydroxylase activity in the selected cultures. This kind of indirect assessment of retrovirus-mediated transduction has certain limitations, especially in cultures contaminated with nonparenchymal cells. [The cultures used by Wolff *et al.* (28) contain ≈20% nonparenchymal cells (4), while those used by Ledley *et al.* (29) initially contain an unspecified number of fibroblasts and endothelial cells that apparently diminish over time in culture.] First, it is impossible to accurately determine the efficiency of transduction of parenchymal and/or nonparenchymal cells by this analysis. More importantly, one cannot rule out the persistence of nontransduced hepatocytes due to cross-feeding by *neo*-expressing parenchymal and/or nonparenchymal cells. Definitive documentation of hepatocyte transduction in these systems will require more direct analyses of hepatocyte transduction such as those described in this report.

Ledley *et al.* (29) also comment on the transcriptional activity of the viral LTR in hepatocytes. A comparison of several retroviral vectors revealed that a LTR-based vector was much less efficient at transducing hepatocytes than was a vector that expressed the *neo* gene from an internal thymidine kinase promoter derived from herpes simplex virus. They ascribe the poor transduction efficiency of the LTR-based vector to inefficient transcription from the LTR. This explanation is in disagreement with several related studies. We have shown very efficient expression of LTR-driven β-galactosidase when the BAG virus is introduced into rat, rabbit, or mouse hepatocytes isolated from adult animals. In addition, Jaenisch studied the infection and expression of replication-competent ecotropic virus introduced into the mouse at different stages of development. Infection of postimplantation embryos and analysis of the resulting adult animals have revealed efficient infection of liver (≈2 proviral copies per diploid genome) as well as the production of high concentrations of viral-specific RNA (30). A more accurate assessment of the relative activity of different promoter/enhancer elements in cultured hepatocytes will require a quantitative analysis of the products of the transduced gene (protein and/or mRNA) in homogeneous populations of hepatocytes.

In summary, we have described a method for introducing new genetic material into primary cultures of adult hepatocytes. This technology should have immediate applications in the study of liver carcinogenesis, regeneration, and gene expression. In addition, the recent development of methods for transplanting hepatocytes (31), together with the efficient transduction of hepatocytes as described in this report, raises the possibility of designing new approaches to liver-directed somatic gene therapy.

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