

Experiments in Transgenic Mice Show That Hepatocytes Are the Source for Postnatal Liver Growth and Do Not Stream

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One hypothesis is that postnatal liver growth involves replication of mature hepatocytes, which have an unlimited proliferative potential. An alternative viewpoint is that only certain periportal cells can replicate extensively and that daughter cells stream slowly from the periportal to the pericentral region of the liver. Transgenic mice expressing the beta-galactosidase (β -gal) gene from the human α 1 antitrypsin promoter were used to examine the proliferative potential of hepatocytes. Surprisingly, only 10% of hepatocytes in two different transgenic lines stain blue with X-gal. In neonatal animals, singlets or doublets of expressing cells are randomly scattered throughout the liver. Although the overall frequency of blue cells is similar in older animals, these cells are present in much larger clusters, suggesting that individual expressing cells have replicated to form a clonally derived cluster. Expression patterns are not altered by the administration of an acute phase stimulus or by the performance a partial hepatectomy, suggesting that the expression state cannot be easily altered, and making it more likely that the expression state is indeed fixed. These results suggest that the clusters of blue cells are clonally derived in the transgenic mice. They argue that the parenchymal hepatocyte is responsible for growth in the postnatal liver and that streaming of liver cells does not occur. (HEPATOLOGY 1995;22:160-168.)

The source of proliferating cells in both the normal and the regenerating liver is controversial. Some investigators favor the hypothesis that all parenchymal hepatocytes are capable of replication regardless of posi-

tion, and that newly formed cells do not migrate. In this model, mature hepatocytes replicate when hepatocyte growth is required to accommodate regeneration or normal liver growth.¹ An alternative viewpoint of liver development is that periportal stem cells give rise to daughter cells that stream toward the pericentral region and undergo apoptosis approximately 1 year later.²⁻⁶ The latter mode of replacement would be similar to what occurs in other epithelial organs such as the skin and intestines, which undergo rapid regeneration in response to a continuous loss of cells.⁷

Our lab is interested in transferring genes into hepatocytes for the purpose of gene therapy. The long-term success of gene therapy requires that either long-lived cells or stem cells be modified. For this reason, it is essential to determine the proliferative potential and longevity of the mature hepatocyte. Although lineage relationships have been determined in rapidly replicating organs such as the gastrointestinal tract by labeling cells with tritiated thymidine or bromodeoxyuridine (BrdU), such experiments are more difficult to perform in slowly replicating organs such as the liver. Indeed, it is the contradictory results obtained with DNA labeling studies^{2-3,8-12} that have led to the mutually exclusive hypotheses regarding liver growth that are mentioned above. We therefore have used an alternative method to mark individual hepatocytes and follow their development over time. Others have used transgenic mice that express a gene in a subset of cells to make conclusions regarding lineage relationships in a variety of organs, including the gastrointestinal tract¹³ and the muscle.¹⁴ We have used such a transgenic mouse line in which the human α 1 antitrypsin (hAAT) promoter directs expression of beta-galactosidase (β -gal) in a subset of liver cells¹⁵ to study the proliferative potential of the differentiated hepatocyte. We note that individual cells can grow into larger clusters. The size of the clusters has no relationship with the liver architecture. We hypothesize that clusters are clonally derived, which suggests that parenchymal hepatocytes located throughout the hepatic lobule are responsible for replication throughout postnatal development. These data argue against the theory of the streaming liver.

MATERIALS AND METHODS

Transgenic Mouse Lines. Transgenic mice containing the 1.2 kb human α ₁-antitrypsin promoter upstream of the 3.5 kb *E. coli lacZ* gene (β -gal) were generated in C57BL/6 inbred

Abbreviations: BrdU, bromodeoxyuridine; β -gal, beta-galactosidase; PCR, polymerase chain reaction; hAAT, human α 1 antitrypsin; PBS, phosphate-buffered solution; TE, Tris-EDTA; FACS, fluorescence activated cell sorting; FDG, fluorescein-di- β -galactoside; FU, fluorescence units; EDTA, ethylene diaminetetraacetic acid; apo-VLDL-II, very low density apolipoprotein II; LPS, lipopolysaccharide; uPA, urokinase plasminogen activator.

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mice, as previously reported.¹⁵ Animals from two independent lines, 3031 and 3035, were identified to contain blue hepatocytes after X-gal staining of liver sections in all offspring that contained transgenic DNA. These animals were bred to generate F₂ and later generation mice, which were screened for the presence of the transgene at 3 weeks of age or later by the performance of biopsy and X-gal staining, or by isolating tail DNA and performing the polymerase chain reaction (PCR) with oligonucleotides specific for the β -gal gene. Homozygous animals were identified by their ability to transmit the transgene to 100% of more than 10 offspring. To obtain heterozygous fetal or perinatal mouse livers, a homozygous male transgenic mouse was mated with a non-transgenic C57BL/6 female overnight, then rotated to a new cage. If pregnancy resulted, it was possible to identify the exact day of conception. To obtain young homozygous animals for analysis, homozygous parents were mated. For analysis of prenatal animals, the mother was anesthetized with inhaled methoxyflurane, killed, and fetuses were collected and frozen in OCT. For neonatal animals, cages were checked every day to determine the exact date of birth, and neonatal pups sacrificed at various days thereafter by decapitation, and the liver frozen in OCT for later analysis. Animal care was in compliance with institutional and NIH guidelines.

X-gal Staining of Mouse Liver Sections. Biopsy samples were obtained during anesthesia with inhaled methoxyflurane. The right lateral lobe was ligated at the pedicle, removed, and immediately frozen in OCT in liquid nitrogen and then at -70°C until sectioned. For mice less than 3 weeks of age, the entire mouse liver was obtained and frozen in OCT. 8- μm sections were fixed with 1.25% glutaraldehyde for 10 minutes at 4°C in phosphate-buffered saline (PBS) containing 139 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L sodium phosphate, and 1.5 mmol/L potassium phosphate at pH 7.2, then stained overnight with a solution containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), 44 mmol/L HEPES pH 7.5, 3 mmol/L potassium ferrocyanide, 3 mmol/L potassium ferricyanide, 15 mmol/L NaCl, and 1.3 mmol/L MgCl₂ as described previously.^{15,16} Sections were counterstained with eosin and coverslips applied. In order to better quantitate the cluster size in animals of various ages, the number of cells per cluster was counted for over 100 events, and an average cluster size \pm SEM was calculated. The average 2-D cluster size was used to calculate the average total volume using the following formula, which assumes that an individual 2-D cluster is a randomly obtained cross-section of a sphere of cells:

$$\text{Average Area} = \frac{\int_0^R \Pi(\text{Radius}^2 - x^2)dx}{\text{Radius}} = \frac{2\Pi\text{Radius}^2}{3}$$

$$\text{Total Volume} = \frac{\sqrt{2\sqrt{3}}\text{Average Area}^{3/2}}{\sqrt{\Pi}}$$

$$= 1.382\text{Average Area}^{3/2}$$

Isolation of Tail DNA and PCR for the β -gal Transgene. PCR¹⁷ of tail DNA was used to determine if some of the animals contained the transgene. A 1-cm or shorter section of a mouse tail was digested overnight with 750 μL of a solution containing proteinase K (Sigma, St. Louis, MO) at 625 $\mu\text{g}/\text{mL}$, and phenol was extracted as described,¹⁸ and resuspended in 10 mmol/L Tris, pH 8.0, 0.1 mmol/L ethylenediaminetetraacetic acid (TE). The oligonucleotides used for PCR were identical to the top strand of the *lacZ*¹⁹ gene at position 1249-1273 (5'GGCATGGTGCCAATGAATCGTCTGA 3') and the

bottom strand at position 1645-1621 (5' TAGCGAAACCGC-CAAGACTGTTACC 3'). PCR was performed on \sim 100 ng of genomic DNA in buffer containing 10 mmol/L Tris-HCl (pH 8.3 at 25°C), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, .01% gelatin, 200 $\mu\text{mol}/\text{L}$ dNTPs, 1 $\mu\text{mol}/\text{L}$ oligonucleotides, and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). Thirty cycles were performed, with denaturation for 30 seconds at 94°C , annealing for 30 seconds at 60°C , and extension for 1 minute at 72°C . DNA was electrophoresed on a 2% agarose gel and observed for the presence of a 396-bp band. All positive animals were later confirmed to have the transgene by performing X-gal staining of a liver biopsy specimen. DNA was isolated in a room devoid of plasmid DNA, and extreme caution was taken to avoid cross-contamination of samples.

Fluorescence-Activated Cell Sorting for β -gal Activity. Hepatocytes with a viability of $>90\%$ were isolated using the collagenase perfusion method of Berry and Friend²⁰ as described previously in detail.¹⁵ Fluorescence activated cell sorting (FACS) for β -gal activity was performed as described.^{21,22} Hepatocytes were resuspended in staining medium (PBS with 4% fetal calf serum and 10 mmol/L HEPES, pH 7.3) at a concentration of 10^7 cells/mL. A solution containing 2 mmol/L of fluorescein-di- β -galactoside (FDG; Molecular Probes, Inc. Eugene, Oregon) in water was photobleached with a 488-nm argon laser shortly before adding to the cells. Fifty microliters of cells was mixed with 50 μL of 2-mmol/L FDG for exactly 1 minute at 37°C , then 1.8 mL of cold staining medium were added, and the cells were kept on ice until FACS was performed (less than 1 hour). Fluorescence intensity of cells was measured as relative fluorescence units (FU).

Acute Phase Stimulation of Mice. Biopsies were performed on transgenic mice at 4 to 6 weeks of age, and liver biopsy specimens of the right lateral lobe were sectioned and stained with X-gal to determine the pattern of expression of the transgene. Performance of the biopsies removed \sim 25% of the liver mass. After allowing 1 month for the liver to recover, animals were treated with trichloroacetic acid precipitated lipopolysaccharide from the 0111:B4 *E coli* serotype (Sigma Chemical, St. Louis, MO) at a dose of 5 mg/kg intraperitoneal²³ in order to induce an acute phase response. Animals were then killed at various times thereafter, and livers were harvested and frozen immediately in OCT.

Southern Blot of Mouse Liver DNA. Livers and spleens were obtained from control and transgenic mice several months after birth, and immediately frozen in liquid nitrogen. Organs were homogenized with a Wheaton type B glass Dounce in PBS containing 10 mmol/L and 0.5% SDS, and digested with 100 $\mu\text{g}/\text{mL}$ proteinase K overnight at 50°C as described,¹⁸ phenol extracted, ethanol precipitated, treated for several hours with RNase A at 50 $\mu\text{g}/\text{mL}$ (U.S. Biochemicals; specific activity 2,557 Units/mg), extracted with phenol, ethanol precipitated, resuspended in 1 mL of TE, and quantitated by optical absorbance at 260 nm. Ten μg of DNA was digested overnight with 50 units of each restriction enzymes, electrophoresed on a 1% agarose gel,¹⁸ stained with ethidium to document that equal amounts of DNA were loaded, and transferred¹⁸ to a Ba-S NC membrane, which is a supported nitrocellulose filter (Schleicher and Schuell, Keene, NH). Blots were hybridized with an RNA probe derived from the 5' end of the hAAT promoter, which was generated as follows. The 1.2-kb hAAT promoter²⁴ was cloned into pGEM4 (Promega) to create hAAT-GEM4-E. This was digested with *Bgl* II, which cuts at -347 relative to the transcription initiation site. T7 RNA polymerase¹⁸ was used to generate an 850-bp

probe with specific activity of 2.5×10^9 cpm/ μ g, which is specific for nt -1,200 to -347 of the hAAT promoter. This region of the hAAT promoter has little homology with the mouse AAT promoter.²⁵ Membranes were hybridized at 42°C with 50% formamide using buffers recommended by the manufacturers, then washed with a final stringency of 15 mmol/L NaCl with 15 mmol/L sodium citrate pH 7.0 (0.1× saturated sodium citrate) at 65°C for 60 minutes.

RESULTS

The relatively liver-specific human α_1 -antitrypsin (hAAT) promoter was cloned upstream of the gene for *E. coli* β -galactosidase (β -gal) and used to generate transgenic mice by oocyte injections, as previously reported.¹⁵ Two founder mice designated 3031 and 3035 were identified to express the transgene by using X-gal staining of liver biopsies. These founders were bred to give non-mosaic progeny whose liver cells presumably all contain the transgene. As previously reported, one remarkable finding is that only 10% of the parenchymal hepatocytes are positive, suggesting that 90% have either no or low level expression. The second surprising finding is that expressing cells are present in clusters of up to 10 to 20 cells, which suggests that they may be derived from a single cell.

Developmental Analysis of X-gal Expression Shows That the Cluster Size Increases Progressively. The fact that expressing cells are located in clusters located randomly throughout the hepatic lobule suggests that expressing cells are clonally derived. If that is the case, the cluster size should increase with age. We performed X-gal staining on livers from F₄ or later-generation transgenic hAAT- β -gal mice in order to determine the pattern of expression at different times of development. Heterozygous animals were studied at either day 1 after birth or at late adult times, as shown in Fig. 1. The heterozygous mice from line 3031 contain 5 to 10 copies of the transgene on an autosomal chromosome, and generally demonstrate expression in 10% of the cells. At day 1 after birth, expressing cells appear as single cells or doublets which are closely spaced and are randomly located throughout the liver architecture, as shown in panel A and B. Analysis at earlier times (embryonic day 18, 19, or 20) showed either no blue cells or occasional singlets (data not shown). When a 4-month-old heterozygous animal was studied, cells were present in much larger clusters that were separated by larger regions of non-expressing cells. The homozygous animals contain the transgenes on both chromosomes, and exhibit expression in ~50% of cells. They similarly exhibited an increase in cluster size with age, although cluster size could not be accurately quantitated because of the high frequency of positive cells (data not shown).

In order to better quantitate the cluster size in animals of various ages, the average number of cells per cluster was determined. Fig. 2A shows that the average two-dimensional cluster size of blue cells in heterozygous transgenic mouse livers increases progressively from day 1 to late adult life. The average 2-D cluster size was used to calculate the average total volume, as described in detail in the Materials and Methods sec-

tion. For each animal, the approximate weight for a C57BL/6 mouse of that age²⁶ was plotted against the calculated total volume of a spherical cluster, as shown in Fig. 2B. The calculated rate of growth of an average spherical cluster of cells is directly proportional to the rate of growth of the animal, with a linear correlation coefficient $r = .992$ with a $P < .001$. This is what would be expected if indeed the parenchymal liver cell is responsible for all liver growth during postnatal development. This conclusion is further supported by the fact that in all cases the total number of blue cells is constant at ~10% and the location of these clusters seems to be random with respect to the liver architecture. A similar developmental pattern was observed with heterozygous livers from line 3035, which contain two to five copies of the transgene integrated into an autosomal chromosome (data not shown). We conclude that the pattern of expression is most consistent with random activation of expression at ~day 1 after birth, rather than a homogeneous activation of expression followed by extinction. The identification of single expressing cells at early time points and large clusters at later times suggests that the clusters of expressing cells are derived from a single progenitor cell.

FDG-FACS Shows That β -gal is not Expressed in 90% of Hepatocytes. Although X-gal staining suggests that the majority of cells do not express the β -gal gene, a small amount of expression would not be detectable with this relatively insensitive method.^{21,22} To document that expression is indeed absent in the non-blue cells, fluorescence-activated cell sorting was performed after addition of Di- β -D-galactopyranoside (FDG-FACS).²² Hepatocytes were isolated as a single-cell suspension from transgenic and control animals by collagenase perfusion. FDG was introduced into the cytoplasm of these cells by administration of a brief hypotonic shock. If β -gal is present, FDG is metabolized into a fluorescent molecule, which can be detected by FACS. When control hepatocytes are tested with FDG-FACS, there is a single peak of cells with a low level of fluorescence, as seen in Fig. 3A. When transgenic hepatocytes are tested, ~10% of the cells have a high level of fluorescence, showing high levels of β -gal, as shown in Fig. 3B. The remaining cells have a low level of fluorescence which is identical to that seen in nontransgenic hepatocytes. Because this method can detect as few as 10 molecules of β -gal per cell,²¹ this shows that indeed 90% of the cells have no expression of the hAAT- β -gal transgene. Similar results were obtained for hepatocytes isolated from line 3031.

Attempts to Activate Nonexpressing Cells by Addition of an Acute Phase Reactant or by Partial Hepatectomy. The very low density apolipoprotein II (apo-VLDL-II) gene of chickens is expressed in only 1% of hepatocytes in males or prepubertal females. Addition of estrogen, however, recruits the remaining cells to express apo-VLDL-II at high levels.²⁷ We therefore considered that previously nonexpressing hepatocytes might be activated to express the hAAT- β -gal transgene by a physiologically relevant stimulus. If ac-

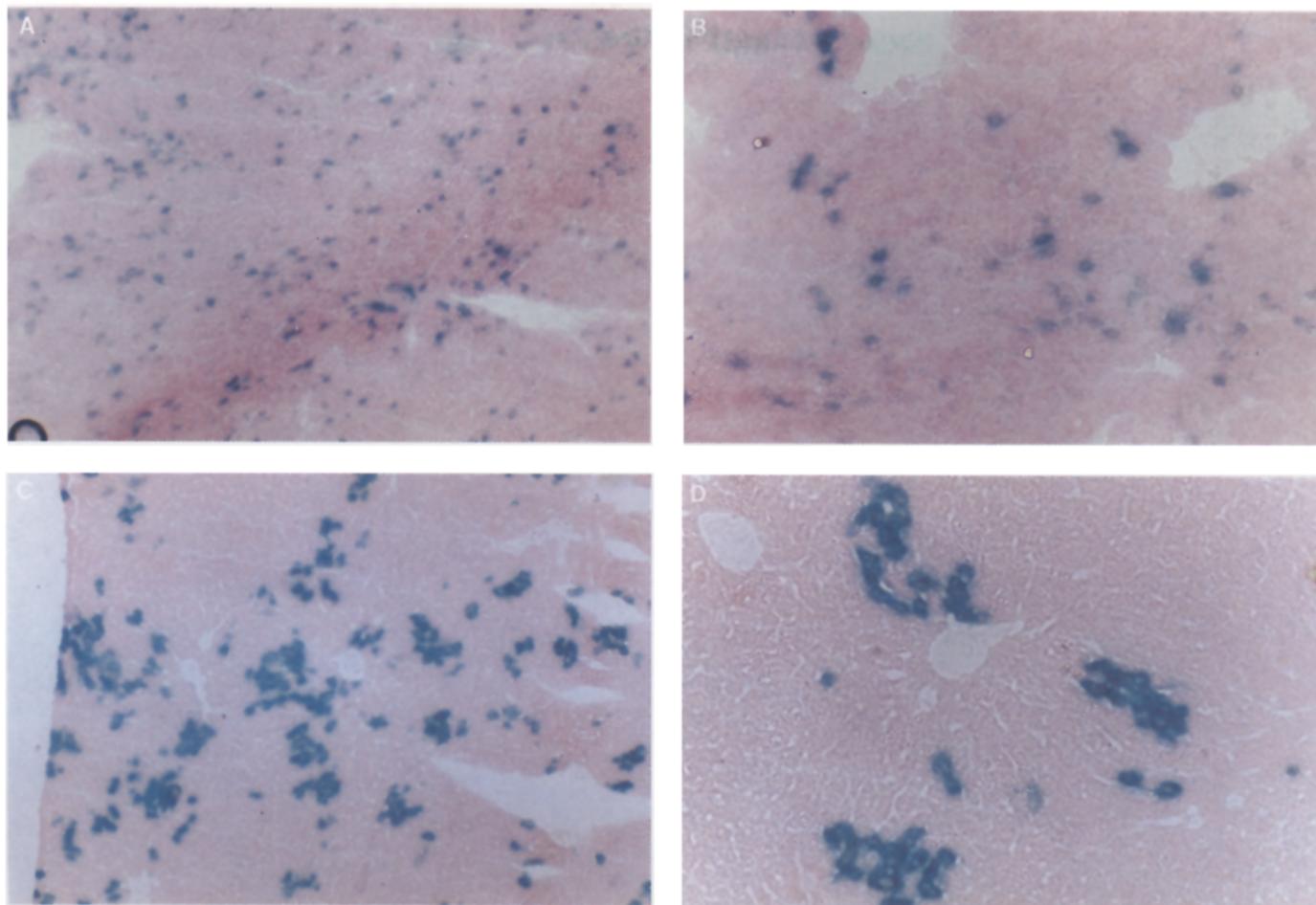


FIG. 1. Developmental analysis of the pattern of expression of the transgene in heterozygous mouse livers from line 3031. Livers were obtained from transgenic mice at different developmental stages; 8- μ m sections were stained with X-gal overnight, then observed for blue cells. (A and B) A 4 \times and 10 \times magnification, respectively, of X-gal staining of a mouse liver obtained 1 day after birth from a heterozygous animal. (C and D) A 4 \times and 10 \times magnification, respectively, of X-gal staining of a mouse liver obtained at 4 months after birth of a heterozygous animal. At 1 day after birth the cells are present as single cells or doublets, scattered randomly throughout the liver. In contrast, at 4 months after birth the cells appear as much larger clusters that are still scattered randomly throughout the liver.

tivation could occur, it would argue that the heterogeneous expression observed in the livers could not be used to make conclusions regarding lineage relationships. Because the human AAT promoter is weakly stimulated by acute phase reactants,²⁸ we determined whether the frequency of expressing cells could be increased by the addition of an acute phase reactant. Four heterozygous animals from line 3031 were treated with lipopolysaccharide (LPS) to induce the acute phase response.²³ Animals were killed 16, 20, 24, or 30 hours after LPS administration and the livers harvested. Sections from both the untreated and the acute phase liver of the same animal were analyzed by X-gal staining. Figure 4 shows that the acute phase stimulus did not alter either the frequency or the pattern of blue cells in a liver that was analyzed 24 hours after the addition of LPS; similar results were obtained with the other animals tested.

We also determined whether induction of liver cell replication could result in the activation of previously nonexpressing cells. A 70% hepatectomy was per-

formed to induce hepatocyte replication, then livers were harvested 1, 2, 3, or 4 days later. There was no change in the percentage of blue cells or their pattern (data not shown). Thus, we conclude that neither an acute phase stimulus nor partial hepatectomy could alter the overall frequency of expressing cells. This result is consistent with the interpretation that the expression state is fixed during postnatal development.

Analysis of the Methylation State and the Copy Number of DNA From Livers and Spleens of Transgenic Mice. Permanently inactivated genes are frequently methylated at CpG dinucleotides, while active genes are often unmethylated.²⁹ If indeed the transgene was primarily methylated in the liver, it would support the hypothesis that permanent inactivation had occurred. Alternatively, we considered that somatic deletion of the transgene could lead to loss of expression, as has occurred in a transgenic mouse line expressing the toxic urokinase plasminogen activator (uPA) gene product in liver cells.³⁰ To address these possibilities, the methylation state and DNA copy number of the

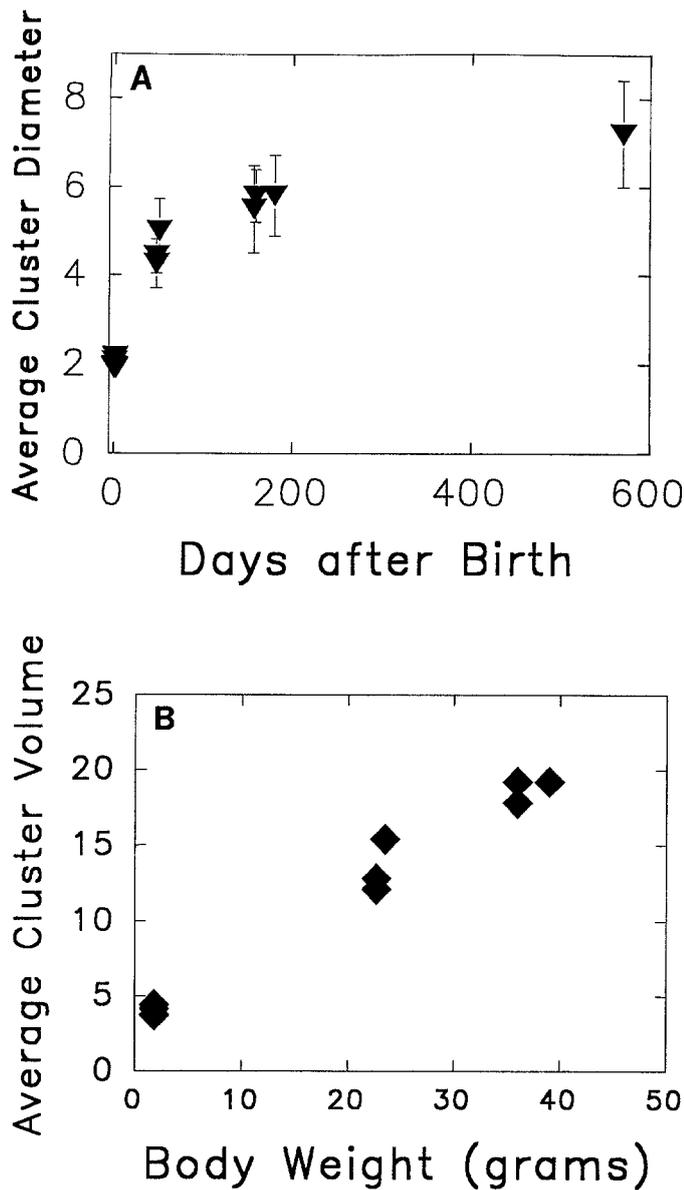


FIG. 2. Quantitation of the cluster size of blue cells at various times after birth. (A) Liver samples obtained from heterozygous animals of line 3031 at various times after birth were sectioned and stained with X-gal, as shown in Fig. 1. The average cluster size \pm SEM was calculated. Each point represents an individual animal. (B) The average cluster size obtained in A was used to calculate the number of cells present in a 3-D cluster as described in the text. This was plotted against the weight for an animal of that age, using data obtained by Poiley.²⁶ There is a linear relationship between the animal weight and the volume of cells in a cluster.

transgene in both the partially expressing liver and the non-expressing spleen were tested. Figure 5A shows the location of restriction sites in the hAAT promoter.²⁴ *Bam*H I sites are present at -46 and -1200 bp relative to the transcription initiation site; *Bam*H I is unaffected by the methylation state of the DNA. The recognition site for both *Hpa* II and *Msp* I is the sequence CCGG, which is present at nucleotide -150 . Methylation inhibits cleavage by *Hpa* II but does not effect

cleavage by *Msp* I.³¹ Equal amounts of DNA derived from the liver and spleen of heterozygous transgenic mice were digested with either *Bam*H I alone, or with *Bam*H I in the presence of either *Hpa* II or *Msp* I. Cleavage at -150 results in disappearance of the 1.15-kb *Bam*H I band, and the appearance of a new 1.05-kb band; methylation at -150 will prevent the cleavage by *Hpa* II, resulting in maintenance of the 1.15-kb band. When spleen DNA from animals of line 3031 is digested with *Bam*H I and *Hpa* II, $>90\%$ of the DNA is methylated, as shown by the presence of the 1.15-kb band seen in Fig. 5B. In contrast, liver DNA is almost completely unmethylated, as shown by the presence of the 1.05-kb band. Ethidium bromide staining of the

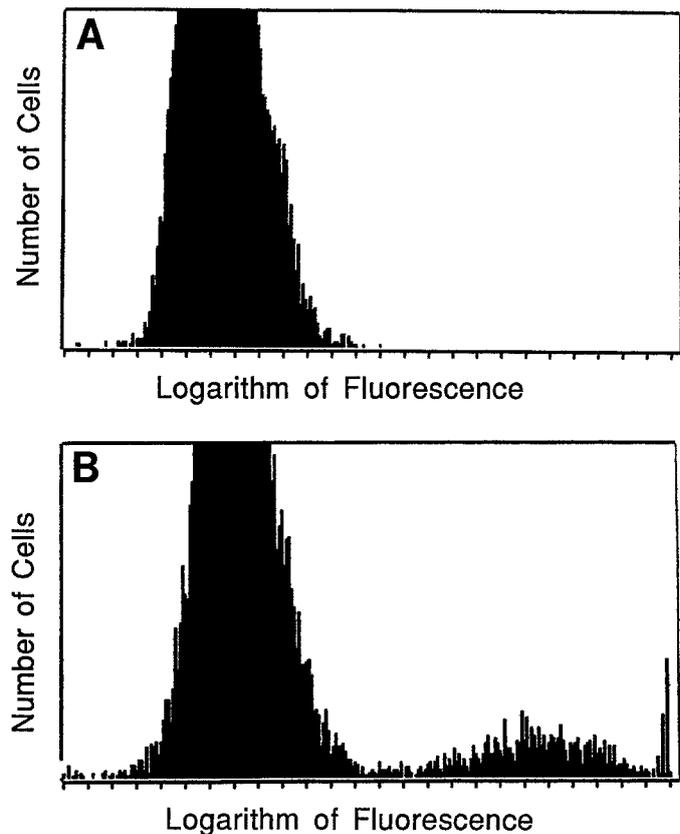


FIG. 3. FACS analysis shows that only 10% of hAAT- β -gal-transgenic mice exhibit any expression of β -gal. (A) FDG-FACS of control nontransgenic hepatocytes. Hepatocytes were isolated by collagenase perfusion, and FDG was delivered to the cytoplasm by performing a brief hypotonic shock. Cells that contain cytoplasmic β -gal metabolize FDG to a fluorescent molecule, which can be quantitated by FACS. The logarithm of the relative level of fluorescence is shown in the X-axis, and the number of cells with that particular level of fluorescence is shown in the Y-axis. Nontransgenic hepatocytes have a single peak of cells with relatively low amounts of fluorescence. (B) FDG-FACS of hAAT- β -gal transgenic hepatocytes from line 3035. Isolated hepatocytes from a 9-month-old transgenic mouse were subjected to FDG-FACS as described in A. Ten percent of the cells have a high level of fluorescence indicating expression of the hAAT- β -gal transgene. The remaining cells have a low level of fluorescence, which is identical with the level observed in the nontransgenic hepatocytes. This indicates that nonblue cells do not express the transgene at low levels.

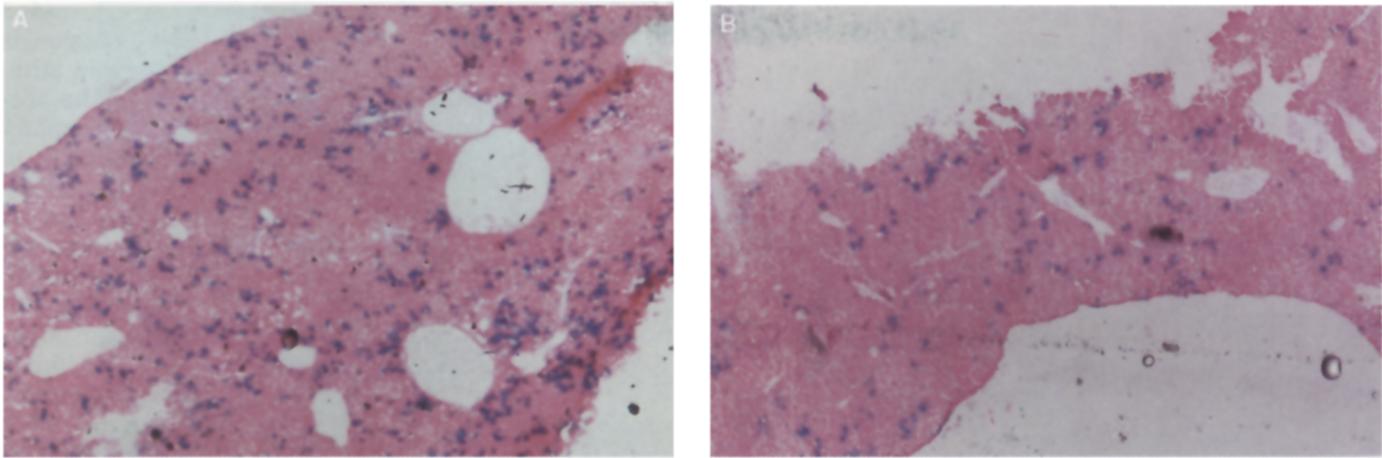


FIG. 4. Induction of an acute phase response does not increase the percentage of cells that express the transgene. An animal from line 3031 had a biopsy performed on it at 2 months of age. One month later, the animal received an intraperitoneal injection of 5 $\mu\text{g/g}$ LPS. Twenty-four hours later the animal was killed, and the liver was harvested. (A) X-gal staining of the liver sample obtained before the induction of the acute phase response. (B) the result of X-gal staining 24 hours after the induction of the acute phase response. There is no change in the frequency of expressing cells, suggesting that an acute phase response cannot activate nonexpressing cells.

DNA samples showed that the total signal in each lane is proportional to the amount of DNA present, making it impossible that 90% of the cells had lost expression because of deletion of the transgene. Similar results were observed for DNA from spleens and livers of line 3035, where the transgene signal was equal in both tissues and was primarily unmethylated in the liver, as shown in Fig. 5C. We conclude that neither somatic deletion nor DNA methylation can explain the loss of expression observed in 90% of the cells.

DISCUSSION

The source of proliferating cells in the normal liver is controversial. Some investigators think that parenchymal hepatocytes account for growth of a normal liver after birth,¹ whereas others hypothesize that stem cells and/or parenchymal hepatocytes located in the periportal region give rise to daughter cells that stream slowly toward the central vein.²⁻⁶ We report here a method of "marking" hepatocytes that has been used to examine the proliferative potential of the differentiated liver cell. hAAT- β -gal transgenic mouse lines were developed previously to use in hepatocyte transplantation experiments.¹⁵ Although expression was observed in the liver, but not in a variety of other organs including the spleen as expected, we were puzzled to observe that two different transgenic lines exhibited expression in only ~10% of all hepatocytes. We reasoned that these transgenic mouse lines might serve as useful tools to learn more about the mechanism of growth of the post-natal liver. Indeed, other investigators have noted a mosaic expression of transgenes, and have used the expression patterns observed to study lineage relationships.^{13,14} A developmental analysis of β -gal expression in hAAT- β -gal transgenic mice demonstrated that single expressing cells were present at 1 day after birth, but much larger clusters of cells were observed at late

time points. We hypothesize that clusters of cells were derived from a single cell, leading us to conclude that it is the parenchymal hepatocyte is responsible for replication in the liver after birth. Because both the single expressing cells and the larger clusters showed no predilection for the periportal or pericentral region, we conclude that cells throughout the liver are all capable of replication, and that streaming of liver cells does not occur. The major caveat to our conclusion is that we cannot conclusively show that the expression state is immutable. Although the FDG-FACS analysis demonstrates that 90% of cells exhibit no expression of the transgene whereas 10% of cells have a high level of β -gal, it is theoretically possible that an individual cell might fluctuate between the on and the off state.

We considered possible mechanisms by which hepatocytes might switch between the off and the on state, and concluded that none were likely to account for the heterogeneity in expression observed. First, a number of genes exhibit a gradient in expression from the periportal to the pericentral region of the liver. If the position of the cell within the hepatic acinus were responsible for the variation in expression, it might change if the cell migrated, as is hypothesized to occur in the streaming liver model.²⁻⁶ In fact, it has been reported that the PiZ variant of hAAT protein is primarily observed in periportal cells by immunocytochemistry,^{32,33} suggesting that the promoter is more active in this region. However, the livers in these PiZ patients are markedly abnormal, expression of the PiZ protein from the hAAT promoter is panlobular in transgenic mice,³⁴ and hAAT mRNA is observed in all hepatocytes of a normal human liver by using *in situ* hybridization.³⁵ Furthermore, the clusters of transgenic hepatocytes in our experiments are present in all zones of the liver. We therefore conclude that variation in expression throughout the hepatic acinus cannot account for

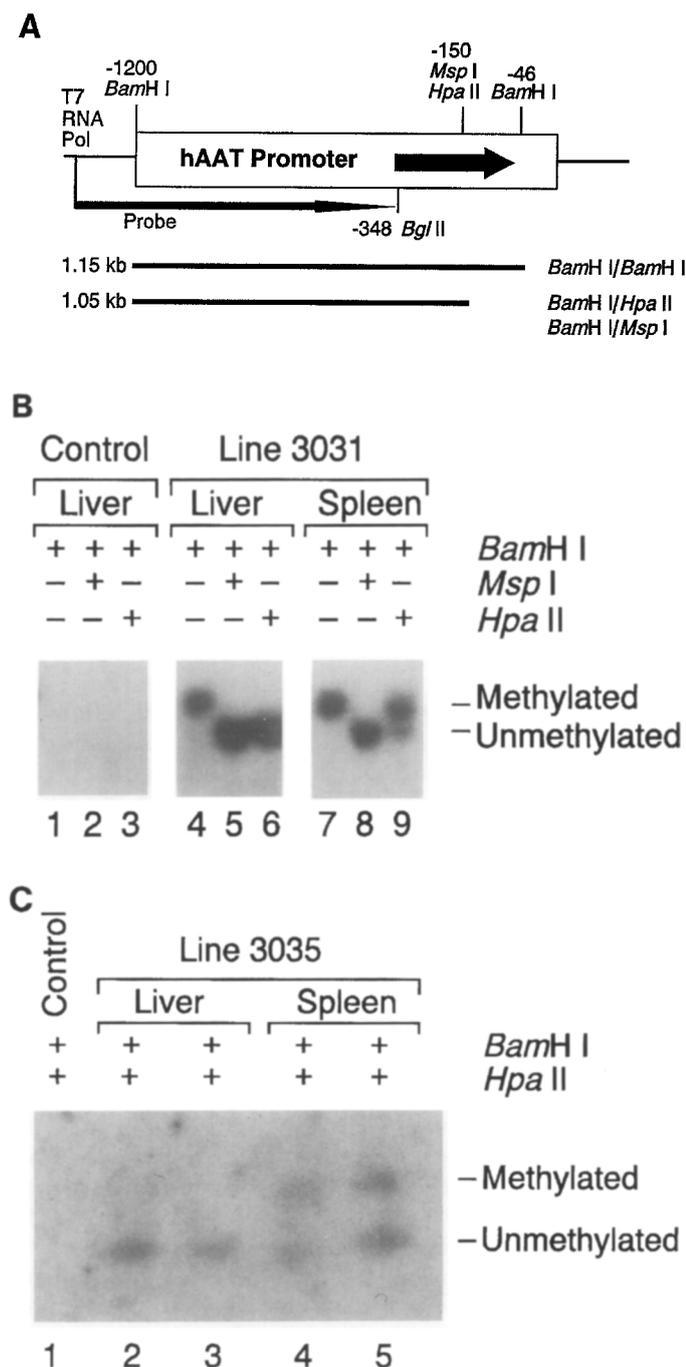


FIG. 5. The transgene is not significantly methylated in the livers of transgenic mice and is present at the same copy number as in the spleen. (A) Schematic diagram of the hAAT promoter. hAAT-GEM3 contains the hAAT promoter cloned into pGEM3. After linearization with *Bgl* II, transcription with T7 RNA polymerase results in an RNA that is complementary to nucleotides -1,200 to -347 of the hAAT promoter. This region has little homology with the mouse AAT promoter. All *BamH* I and *Msp* I/*Hpa* II sites and the expected lengths of DNA after restriction are shown. (B) Southern blot of hepatic and splenic DNA from transgenic mice of line 3031 using a methylation-sensitive restriction enzyme. Chromosomal DNA was isolated from the liver and spleen of transgenic mice at 6 months after birth; 10 μ g was digested with the designated enzymes, and the DNA electrophoresed on a 1% agarose gel. After transfer to a nitrocellulose membrane, the blot was hybridized with an RNA probe specific for nucleotides -1,200 to -346 (relative to the

transcription initiation site) of the hAAT promoter. The 1.15-kb *BamH* I band is designated on the right as "methylated," whereas the 1.05 kb *BamH* I/*Hpa* II-*Msp* I band is designated as "unmethylated." Lanes 1, 4, and 7 contain DNA that was digested with *BamH* I only (methylation insensitive). Lanes 2, 5, and 8 contain DNA that was digested with *BamH* I and *Msp* I (methylation insensitive), and marks the position of the 1.05-kb *BamH* I/*Hpa* II-*Msp* I band. Lanes 3, 6, and 9 contain DNA that was digested with *BamH* I and *Hpa* II (methylation sensitive), and can be used to estimate the percentage of DNA that is methylated. DNA is derived from a control non-transgenic liver (lanes 1-3), transgenic mouse liver (lanes 4-6), and transgenic mouse spleen (lanes 7-9). The liver DNA is less than 5% methylated, whereas the spleen DNA is ~90% methylated. (C) Southern blot of liver and spleen DNA from transgenic mice of line 3035 using methylation sensitive restriction enzymes. DNA from a control liver or from line 3035 transgenic mouse livers or spleens was treated as described in B. The position designated unmethylated identifies the 1.15-kb *BamH* I band, whereas the position marked unmethylated identifies the 1.05-kb *BamH* I/*Hpa* II-*Msp* I band. The samples shown in all lanes were digested with both *BamH* I and *Hpa* II. Lane 1 shows DNA from a nontransgenic littermate, lanes 2 and 3 show DNA from transgenic mouse livers, whereas lanes 4 and 5 show DNA from transgenic mouse spleens. The transgenic mouse liver DNA is <5% methylated, whereas the spleen DNA is ~50% methylated.

the heterogeneous expression observed here. A second possible explanation for heterogeneous expression is that some cells require a physiologically relevant stimulus to be activated to express. For example, the apoVLDL-II gene is expressed in only a subset of hepatocytes in male or prepubertal females, and addition of estrogen results in recruitment of additional cells to the expression state.²⁷ Because hAAT is a weak acute phase reactant whose expression increases about twofold in humans under stress, we considered that addition of an acute phase stimulus might increase the percentage of expressing cells in the transgenic mice. Neither addition of an acute phase stimulus nor induction of replication by performing a partial hepatectomy could increase the percentage of expressing cells, however. A third possible reason for the loss of expression is that either somatic deletion or methylation of the transgene occurred. Indeed, Sandgren et al reported that somatic deletion of a transgene was responsible for the appearance of normal hepatocytes in mice whose livers expressed the toxic urokinase plasminogen activator gene product.³⁰ Somatic deletion of the transgene cannot account for the loss of expression in the hAAT- β -gal mice, however, as the DNA copy number in the liver was equal to that in the spleen. Similarly, methylation does not appear to be responsible for the loss of expression, as liver DNA was primarily unmethylated, although the technique used here only assessed the methylation state at the *Hpa* II site at -150 bp and may not reflect the methylation state at other CpG sites.

Although the biochemical reason why 90% of hepatocytes fail to express the hAAT- β -gal transgene is unclear, the difference in the frequency of expressing cells between homozygotes (both chromosomes have the gene, and ~50% of hepatocytes are blue) and heterozy-

gotes (one chromosome has the gene, and ~50% of hepatocytes are blue) is not clear. The difference in the frequency of expressing cells between homozygotes and heterozygotes could be due to a number of factors. First, the frequency of expressing cells could be due to a difference in the number of transgene copies. Second, the frequency of expressing cells could be due to a difference in the methylation state of the transgene. Third, the frequency of expressing cells could be due to a difference in the expression level of the transgene. Fourth, the frequency of expressing cells could be due to a difference in the stability of the transgene. Fifth, the frequency of expressing cells could be due to a difference in the regulation of the transgene. Sixth, the frequency of expressing cells could be due to a difference in the response of the transgene to environmental factors. Seventh, the frequency of expressing cells could be due to a difference in the response of the transgene to genetic factors. Eighth, the frequency of expressing cells could be due to a difference in the response of the transgene to epigenetic factors. Ninth, the frequency of expressing cells could be due to a difference in the response of the transgene to stochastic factors. Tenth, the frequency of expressing cells could be due to a difference in the response of the transgene to unknown factors.

gotes (one chromosome has the gene, and ~10% of hepatocytes are blue) makes it unlikely that some cells fail to express the gene because they lack critical transcription factors, and suggests instead that activation (or less likely inactivation) is a stochastic event. Furthermore, it is impossible to activate an extinguished chromosomal hAAT gene in somatic cell hybrids by transfection of the necessary liver-specific transcription factors with (Bulla G, Personal communication) or without³⁶ the addition of inhibitors of methylation. This implies that something other than methylation is responsible for preventing the chromosomal hAAT promoter from being activated by the same transcription factors that were able to activate the hAAT promoter when introduced as a plasmid.³⁶ These experiments show that it is difficult to activate an extinguished hAAT gene, making it more likely that nonexpressing cells will remain inactive. Thus, for a variety of reasons we feel it is unlikely that the expression state changes in hepatocytes from the hAAT- β -gal mice. Indeed, the progressive increase in 3-D cluster volume is quite consistent with the rate of growth of the total animal, which strongly suggests that differentiated hepatocytes are dividing to provide more cells. In addition, heterogeneous expression of transgenes has occurred in a variety of organ systems such as muscle¹⁴ or the gastrointestinal tract,¹³ and the islands of expressing cells in these experiments are clearly clonally derived.

Implications for Liver Development. The streaming liver theory²⁻⁶ states that cells located in the periportal region of the liver replicate, then progeny migrate toward the pericentral region and undergo apoptosis after approximately 1 year. Although the streaming liver theory has a number of disciples, there are only two pieces of data that support it. First, tritiated thymidine labeling in either normal livers^{2,3,8} or livers that are replicating in response to partial hepatectomy⁹ shows that labeled cells appear to migrate from the periportal to the pericentral region over time. However, a number of other investigators report no differences between the frequency of labeling in various regions of the hepatic lobule in normal livers and do not see migration over time.¹⁰⁻¹² It is likely that the low frequency of labeling of hepatocytes due to their slow replication rate³⁷ and problems of label reutilization³⁸ account for this discrepancy. A second fact that is cited by supporters of the streaming liver theory is that several liver-specific genes exhibit a gradient in expression from the periportal to the pericentral region.⁴ However, alternative explanations exist to explain the gradients noted in expression of genes throughout the liver acinus, such as metabolic variation.

Although there are no data that unequivocally support the streaming liver theory, there are a great deal of data that support the alternative theory that all hepatocytes are capable of replication regardless of their position. First, a partial hepatectomy induces one^{9,12} or more³⁹ rounds of replication of most hepatocytes within 48 hours, implying that all have proliferative potential. Indeed, we have performed retroviral transduction of

rat hepatocytes *in vivo* and determined that the cells throughout the liver can be transduced. Furthermore, these marked cells undergo additional rounds of replication in response to repeated hepatectomies (data not shown). Second, experiments in which chimeric livers are created by morula aggregation between histologically distinguishable strains of rats show patches of donor cells whose location is unrelated to the liver architecture and in fact similar to what we observe in the transgenic mice at late time points.⁴⁰ Although expression patterns were not followed at different times of development, the pattern observed is consistent with a computer-generated model that assumes that cells replicate equally well throughout the liver. Additional support for the hypothesis that parenchymal hepatocytes have a high proliferative potential was obtained in an experiment where β -gal expressing hepatocytes underwent up to 12 doublings after transplantation into a recipient mouse whose liver expressed a toxic transgene product.⁴¹ These experiments suggest that mature hepatocytes have a tremendous proliferative potential, although the severely abnormal recipient liver may have altered the developmental pattern of the donor cells and the fact that less than 7% of the injected cells gave rise to nodules raised the possibility that rare stem cells and not parenchymal cells were the cells that proliferated extensively. Similarly, Chen et al⁴² noted that adult hepatocytes from either the periportal or the pericentral region were able to proliferate extensively after transplantation into the rat spleen, suggesting that all hepatocytes are capable of replication regardless of position in the liver. Finally, Bralet et al⁴³ have used retroviral marking to label cells and follow their development over time. Because their β -galactosidase marker gene was expressed long-term, expression could be evaluated 1 year after transduction occurred. They observed that individual hepatocytes could grow into larger cluster of cells, and there was no evidence of migration over time.

Thus, our data from transgenic mice are consistent with a variety of other experiments that strongly support the hypothesis that the parenchymal hepatocyte gives rise to more hepatocytes in the liver under most circumstances, although it is possible that stem cells⁴⁴ play an important role in some circumstances, such as regeneration in response to hepatotoxins. The implication of our conclusion is that the transfer of genes into differentiated hepatocytes should lead to permanent correction of a genetic deficiency. Indeed, we have followed rats that were transduced with a retroviral vector directing expression of the hAAT serum protein, and observed stable expression for 2 years (Ponder K, Unpublished data). The second implication of our results is that the parenchymal hepatocyte represents a highly differentiated cell type that is able to change into a proliferative state if necessary. This differs from a number of other organs, such as the skin, the gastrointestinal tract, and the bone marrow, where highly differentiated cells cannot return to a rapidly proliferating population.⁷

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