

Cell Lineage Study in the Liver Using Retroviral Mediated Gene Transfer

Evidence against the Streaming of Hepatocytes in Normal Liver

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The fate of normal hepatocytes in adult rat liver was studied after genetic labeling using the Escherichia coli β -galactosidase gene coupled to a nuclear localization signal. The marker gene was introduced by direct in vivo retroviral-mediated gene transfer into hepatocytes 24 hours after partial hepatectomy. Analysis of β -galactosidase expression in the liver at various time after gene transfer revealed that labeled hepatocytes were distributed throughout the entire lobule with a predominance in the periportal and mediolobular regions. Long-term experiments demonstrated that division of hepatocytes did occur as was revealed by the increasing number of β -galactosidase-positive cells in isolated clusters. There was no evidence for the participation of stem cells in this process. Moreover, we found that after more than 1 year, the pattern of distribution of positive cells within the lobule was not modified. This suggests that hepatocytes do not migrate from the portal space to the perivenous region, as has been previously hypothesized. (Am J Pathol 1994, 144:896-905)

The liver is considered as a slow-renewing organ, and the life span of hepatocytes has been estimated to be 200 to 400 days.¹ For this reason, little is known regarding the cellular and molecular mechanisms that govern cell turnover in normal hepatic tissue. The cellular integrity of epithelia is usually maintained by the multiplication of stem cells that provide newly formed cells able to mature and replace senescent cells. The

existence and the role of such stem cells in the turnover of adult hepatocytes are still a matter of debate.²

On the one hand, mature hepatocytes retain a replicative potential although they are highly differentiated. Any decrease in the functional liver mass resulting from toxic injury or surgical resection triggers a proliferative response of remnant hepatocytes with no involvement of stem cells. This is particularly striking after a two-thirds hepatectomy in the rat when as much as 30% of hepatocytes are able to enter the cell cycle within 24 hours.³⁻⁵ The regeneration process allows a restitution *ad integrum* of the liver mass in less than 10 days.⁶ It seems therefore that in most situations, the multiplication of mature hepatocytes accounts for the maintenance of liver cellularity, and there is no evidence for a role of stem cells in the regeneration.

On the other hand, during the past years many investigators have pointed out the possible existence of stem cells in the adult liver. Progenitor cells are readily observed during liver ontogenesis, and at embryonic day 12 in the rat, the liver is composed of bipotential progenitor cells committed to differentiate along the hepatocyte or biliary cell lineage.⁷ These liver stem cells that display specific antigenic and enzymatic profiles are no longer detected after birth. However, after massive galactosamine-induced hepatic necrosis⁸ or during the course of carcinogenic treatment, the proliferation of a new population of small so-called oval cells⁹ has been described. Oval cells display antigenic and protein patterns that are strikingly similar to hepatic stem cells¹⁰⁻¹² and have been shown

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to be able to differentiate into hepatocytes.^{13,14} The actual origin of the oval cells is not clearly established, but the evidence suggests that they may arise from cells of the Hering canal^{15,16} or from periductular cells.¹⁷ The findings that suggest the continued presence of stem cells have strengthened the model of the streaming liver. The streaming model predicts that there is a constant production of new hepatocytes at the outer rim of the portal space.¹⁸ These newly formed cells then move along the sinusoid toward the central vein where they are eventually eliminated. During their journey, the hepatocytes mature as they become older and as a consequence display different enzymatic patterns and replicative potential.¹⁹ This model is supposed to explain both the metabolic heterogeneity found in the lobule and the kinetics of cell division following partial hepatectomy. Specialized metabolic functions are not evenly distributed within the hepatic lobule.²⁰ For example, enzymes involved in glycogen metabolism and gluconeogenesis as well as enzymes of ammonia metabolism are found predominantly in the central region of the lobule (zone 3). Such an heterogeneity has been attributed to the concentration gradient of metabolites in the unidirectional blood supply of the acinus or to specific cell-to-cell interactions.^{21,22} According to the streaming model, this heterogeneity might be explained in terms of position-related maturation of hepatocytes.²³ Another example of cell heterogeneity is found during the regeneration that follows partial hepatectomy. The wave of mitosis first involves the periportal region (zone 1) at 24 hours, then moves along the acinus and reaches the pericentral region (zone 3) after 48 hours.^{3,4} In addition, it has been shown that the multiplication of pericentral hepatocytes *in vitro* was delayed after hepatic resection when compared to other parenchymal cells.²⁴ It seems therefore that the actual position of any given hepatocyte within the hepatic lobule dictates its temporal response to proliferating stimuli. According to the streaming model, the pattern of the kinetics of DNA synthesis results from the higher proliferating capacity of young hepatocytes located around the portal space.

In the present study, we have used genetic labeling of hepatocytes *in vivo* to determine the fate of hepatocytes in the aging liver. For this purpose, we have used retroviral vectors because they meet the following criteria required for cell lineage analysis.²⁵ First, infection with recombinant retroviruses results in the stable integration of the marker gene in a low copy number and defined proviral configuration. Such an integration results in the replication of the transgene along with the cellular genome and therefore in the transmission of the label in the progeny of one single

cell. Secondly, the expression of the marker gene is cell restricted. Indeed, only the cells that have integrated the transgene are able to express it, and there is no possibility of reutilization of the label as it has long been demonstrated for [³H]thymidine.^{26,27} We have used a marker gene, the *Escherichia coli* β -galactosidase coupled to a nuclear localization signal (nls),²⁸ which does not disturb or influence the fate of the labeled cell. The appearance of isolated clones of labeled hepatocytes and their increase in size during time as well as their constant distribution throughout the entire lobule led us to conclude that hepatocytes are able to divide and that there is no displacement of cells within the lobule during the life of the animal.

Materials and Methods

Retroviral Vectors

Amphotropic recombinant retroviral vectors carrying the *Escherichia coli* β -galactosidase gene (lac Z) were constructed as previously described.²⁹ We used a modified β -galactosidase gene coupled to a nuclear localization signal of simian virus 40, which targets the protein to the outer membrane of the nucleus.²⁸ The resulting nls lacZ cassette was inserted into the *Bam*H1 site of the pMFG retroviral plasmid. In this construct, the nls lacZ transcription is under the control of the regulatory elements of the viral long terminal repeat (LTR). The pMFG-nls lacZ plasmid was transfected into the ecotropic Ψ CRE packaging cell line.³⁰ Forty-eight hours later the culture medium was harvested, filtered through 0.45- μ filter, supplemented with polybrene (8 μ g/ml), and used to infect amphotropic Ψ CRIP packaging cells.³⁰ Clones of positive Ψ CRIP cells were isolated by fluorescent-activated cell sorting using a fluorescent substrate of β -galactosidase.³¹ The titers of the clones were assayed by infecting NIH 3T3 cells with serial dilutions of the retrovirus-containing culture medium. One of the clones, NB16, was used in this study. Its titer when assayed on NIH 3T3 cells ranged from 5×10^5 to 10^6 focus forming unit/ml.

Asanguineous Perfusion of the Liver

Nine male Wistar rats (150 to 160 g body weight) were subjected to a two-thirds partial hepatectomy according to the procedure of Higgins and Anderson.⁶ The right adrenal vein was ligated in the same surgical step. Twenty-four to 27 hours later, after ligation of the gastroduodenal vein, the liver was temporarily excluded from the circulation by successively clamping

Table 1. *Quantification of Clusters of Different Sizes at Various Times after Labeling*

Number of positive cells/cluster	Number of clusters*		
	3 days	15 days	15 months
1-3	329	285	217
4-6	0	13	33
>6	0	3	45

* For each time point, values represent the total number of clusters of each size in three different animals.

the hepatic artery, the portal vein, and the infrahepatic and suprahepatic vena cava. The liver was then perfused with the retrovirus-containing solution through a silastic canula inserted into the portal vein and the perfusion medium was collected from the infrahepatic portion of the vena cava. Thirty to 40 ml of viral solution containing polybrene (8 µg/ml) were infused at a constant flow rate of 4 ml/minute. After the perfusion, the vessels were sutured and the normal hepatic vascularization was restored. To avoid a high mortality rate associated with a prolonged clamping of the portal blood flow, the whole perfusion procedure was carried out in less than 20 minutes. All surgical procedures were conducted on deeply ether-anesthetized animals according to the guidelines of the French Ministry of Agriculture.

Histochemical Detection of β-Galactosidase Activity

At various times after the perfusion, rats were ether-anesthetized and laparotomized. The portal vein was clamped, and the liver was rinsed *in situ* by perfusion of the portal vein with phosphate-buffered saline (PBS). After washing, fixation was obtained by perfusion with a 4% paraformaldehyde solution prepared in PBS. The livers were removed and cut into 5-mm-

thick sections. Fragments were then immersed overnight in a 30% sucrose solution in PBS and snap-frozen in dry ice-cold isopentane. Cryostat sections (10-µ thick) were then made, incubated for 6 to 15 hours in PBS containing 0.4 mg/ml 5-bromo 4-chloro 3-indolyl βD galactopyranoside (X-Gal), 4 mmol/L potassium ferricyanide, 4 mmol/L potassium ferrocyanide and 2 mmol/L MgCl₂. Sections were counter-stained with hematoxylin and eosin, dehydrated, and mounted.

Quantification and Distribution of β-Galactosidase-Positive Cells

The β-galactosidase-expressing cells appeared with blue-stained nuclei and were either isolated or gathered in groups of adjacent stained cells. The number of β-galactosidase-positive cells in each cluster was counted in X-Gal-stained sections by light microscopic examination at low magnification. The spatial localization of the clusters within the hepatic lobule was determined using the portal space and central vein as references. Using a micrometric ocular, the distance between a portal space and a central vein surrounding each cluster was divided into three equal segments that correspond to zones 1, 2 and 3 respectively. This allowed localization of the cluster of positive cells in one of these three segments.

Three animals were analyzed for each time point (3 days, 15 days and 15 months), and for each animal 90 to 120 clusters were examined in various sections of different lobes and their position determined within the hepatic lobule.

Results

In the normal liver, most hepatocytes are quiescent and therefore not susceptible to retroviral-mediated

Table 2. *Spatial Distribution of the Clusters of Positive Cells within the Hepatic Lobules at Various Time after Labeling*

Time of sacrifice	Rat number	Number of clusters (% of the total)		
		Zone 1	Zone 2	Zone 3
3 days	A1	48 (40%)	48 (40%)	24 (20%)
	A2	49 (40.5%)	40 (33%)	32 (26.5%)
	A3	40 (47%)	30 (32%)	18 (22%)
	mean	(42 ± 4%)	(35 ± 4%)	(23 ± 3%)
15 days	B1	34 (31%)	53 (47%)	25 (22%)
	B2	42 (45%)	26 (28%)	25 (27%)
	B3	41 (43%)	34 (35%)	21 (22%)
	mean	(40 ± 7.5%)	(37 ± 10%)	(24 ± 3%)
15 months	C1	33 (34%)	33 (34%)	31 (32%)
	C2	52 (53%)	25 (26%)	21 (21%)
	C3	38 (38%)	37 (37%)	25 (25%)
	mean	(42 ± 10%)	(32 ± 6%)	(26 ± 6%)

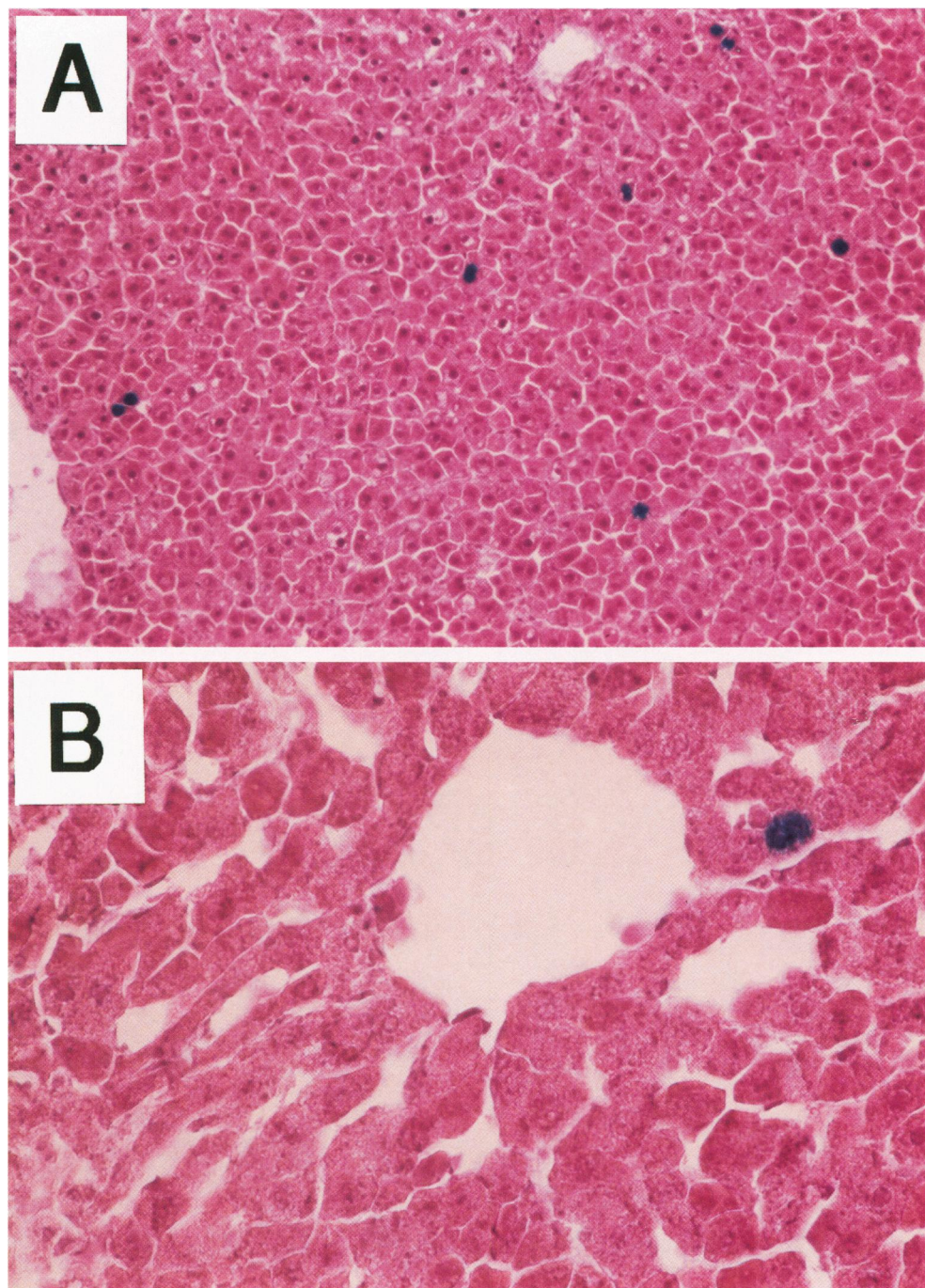


Figure 1. Histochemical detection of β -galactosidase activity in rat liver 3 days after perfusion. Rats were subjected to partial hepatectomy and asanguineous liver perfusion with recombinant retroviral vectors as indicated in Material and Methods. Three days after perfusion, the liver was fixed and cryosections ($10\ \mu$) were stained with X-Gal and counterstained with hematoxylin and eosin. **A:** Overall distribution of the positive cells in the hepatic lobule (magnification: $150\times$). **B:** Positive cells near a central vein (magnification: $600\times$).

gene transfer. Cell division is required to achieve correct integration and expression of retroviral vector genome.^{32,33} We previously demonstrated that asanguineous perfusion with retroviral vectors of the regenerating liver 1 day after partial hepatectomy allowed the expression of the transgene in 1 to 5% of

hepatocytes.²⁹ The delay between hepatectomy and retroviral infection is an important parameter to achieve maximal gene transfer into hepatocytes (Branchereau et al, submitted). When the perfusion with retroviral vectors is carried out more than 36 hours after partial hepatectomy, the number of

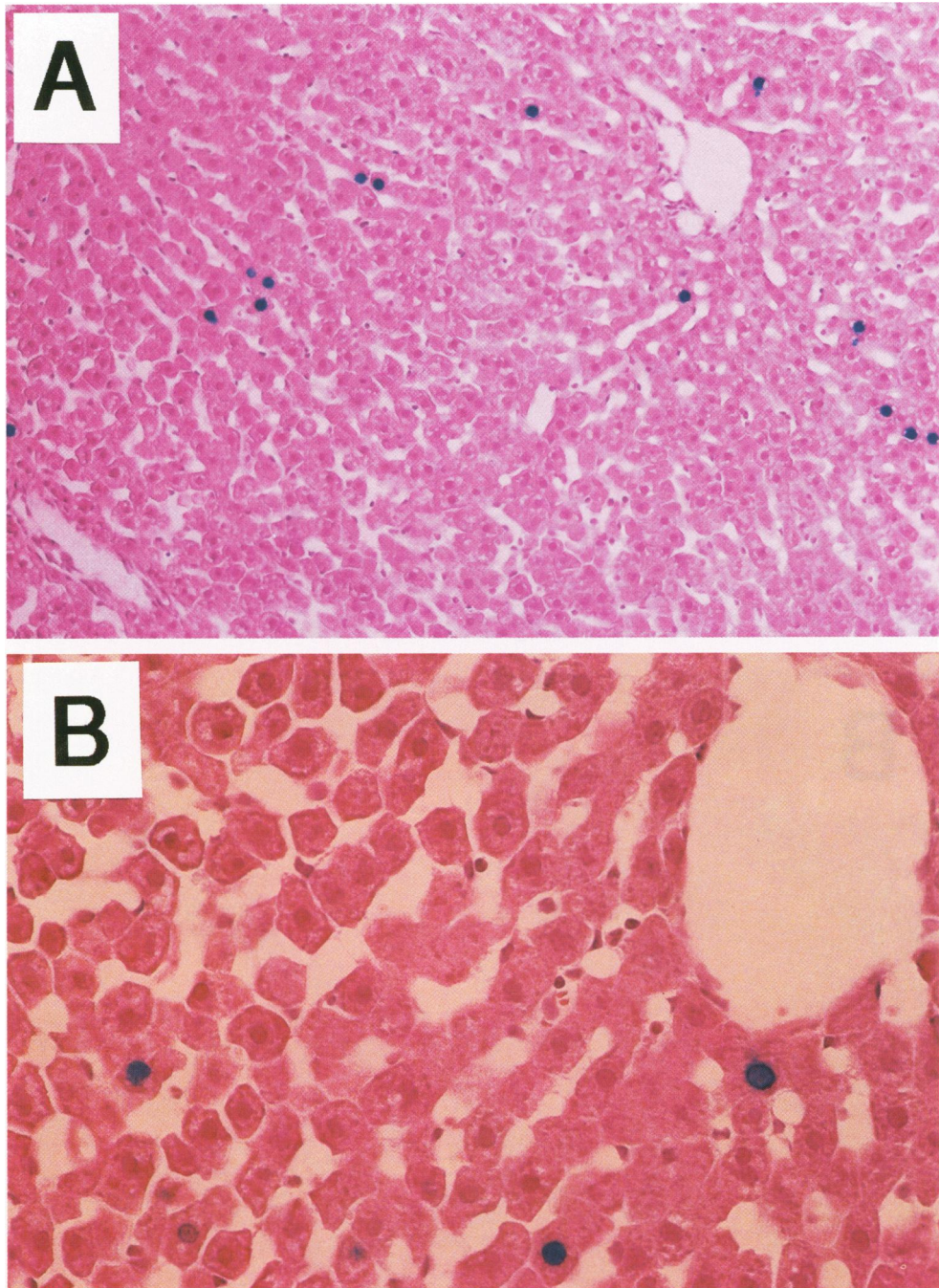


Figure 2. Histochemical detection of β -galactosidase activity in rat liver 15 days after perfusion. Same as Figure 1 except that the animal was sacrificed 15 days after the perfusion. **A:** Overall distribution of the positive cells in the hepatic lobule (magnification: 150 \times). **B:** Positive cells near a central vein (magnification: 600 \times).

β -galactosidase-positive cells decreased dramatically. In addition, the peak of division of nonhepato-cytic cells in the liver following partial hepatectomy occurs 24 hours after the bulk of hepatocyte mitosis.^{3,4} For these reasons, we chose to perfuse the livers between 24 and 27 hours after partial hepate-

ctomy to label hepatocytes specifically and with a maximal efficiency.

On X-Gal-stained cryostat sections, the cells expressing β -galactosidase coupled to the nuclear localization signal are readily detected and appear with a blue nucleus (see figures). Expression of the

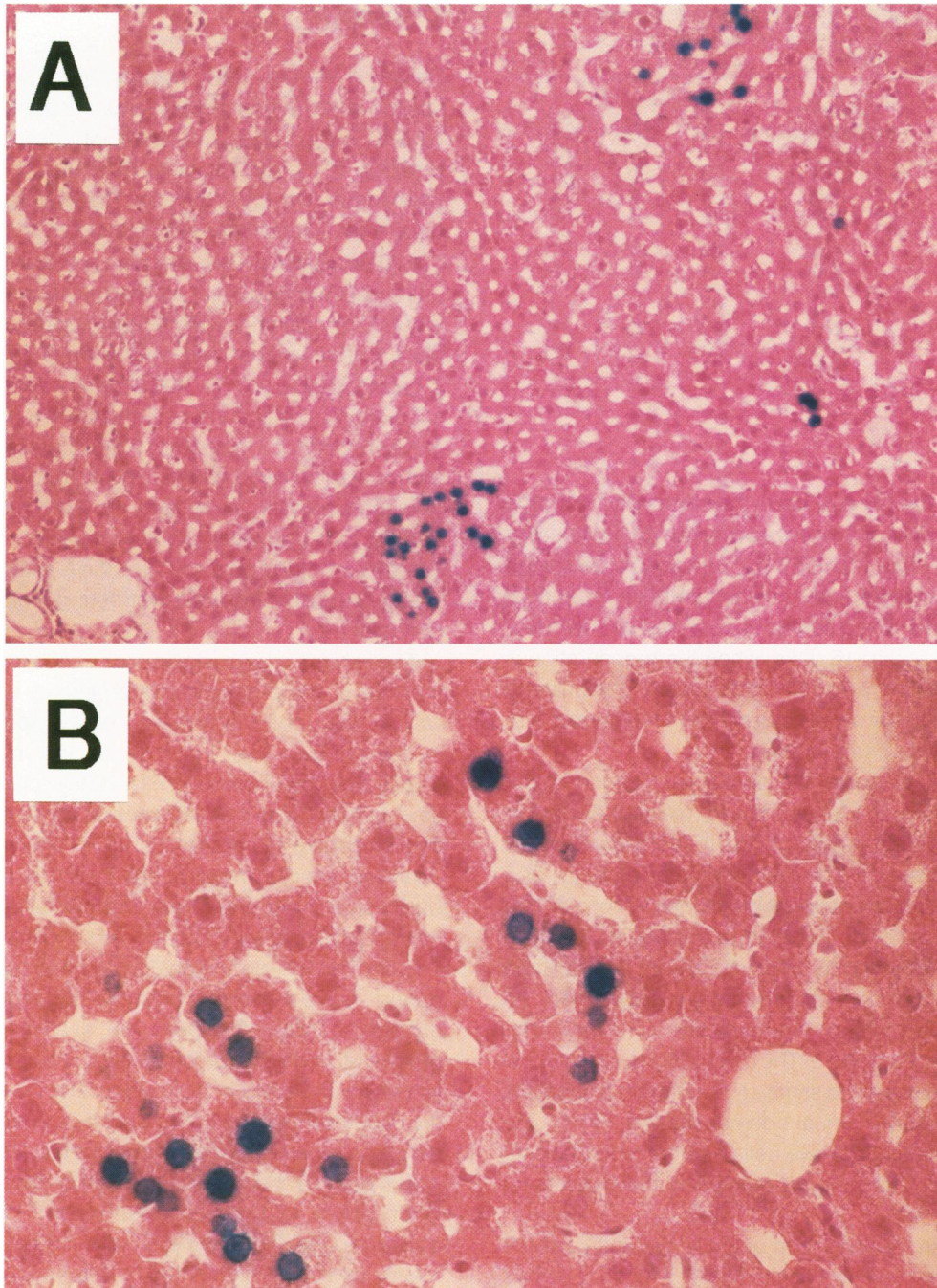


Figure 3. Histochemical detection of β -galactosidase activity in rat liver 15 months after perfusion. Same as Figure 1 except that the animal was sacrificed 15 months after the perfusion. **A:** Overall distribution of the positive cells in the hepatic lobule (magnification: 150 \times). **B:** Positive cells near a central vein (magnification: 600 \times).

β -galactosidase gene does not disturb the overall structure of the cell. Also we did not observe any alteration in the structure of the hepatic parenchyma after retroviral-mediated gene transfer.

We first analyzed the number of β -galactosidase-positive cells per cluster in various sections obtained from different lobes in animals sacrificed 3 days, 15

days, and 15 months after gene transfer (Table 1). The clusters were divided into three groups according to their size (ie, the number of positive cells per cluster): small (one to three positive cells), intermediate (four to six positive cells), and large clusters (seven or more positive cells). Three days after perfusion, all the positive cells were found in clusters of less than three

Table 3. *Spatial Distribution of Clusters of Different Sizes in Hepatic Lobules 15 Months after Labeling*

	Positive cells/cluster	Rat number		
		C1	C2	C3
Zone 1	1-3	22	35	27
Zone 1	4-6	2	8	8
Zone 1	>6	9	8	8
Zone 2	1-3	23	20	21
Zone 2	4-6	4	0	9
Zone 2	>6	6	5	7
Zone 3	1-3	26	19	24
Zone 3	4-6	2	1	1
Zone 3	>6	3	1	0

cells. The size of the clusters then increased with time and in the liver of animals sacrificed 15 months after perfusion, more than 25% of the clusters contained four cells or more, with a maximum of up to 34 cells in one cluster. The differences observed between animals analyzed at 15 days, when the regeneration is fully completed, and 15 months clearly demonstrate that hepatocytes are able to divide in the normal liver during the life of the animal.

We next analyzed the spatial distribution of the clusters within the lobule. The lobule was divided in three zones according to the model proposed by Rappaport et al.³⁴ The periportal, mediolobular, and pericentral region correspond to zone 1, zone 2, and zone 3 respectively. For each animal, 90 to 120 clusters were recorded in different parts of the liver and their localizations within the lobule determined. As shown in Table 2, the positive cells were mainly located in zones 1 and 2. The distribution pattern of the positive cells was the same at the different time points of the study, thus demonstrating that the β -galactosidase-positive cells had not moved inside the lobule during the 15 months of the experiment (Figures 1 to 3).

Finally, in the long-term animals, we analyzed the spatial distribution of the clusters as a function of their size (Table 3). Most of the larger clusters were located in zones 1 and 2. We rarely observed the presence of large clusters of positive cells close to the central vein in zone 3 (Figure 3B). This suggests that the replicative potential of hepatocytes may be greater in zone 1 and 2. Moreover, clusters of β -galactosidase-positive cells were still present very close to the portal space 15 months after the perfusion (Figure 4, A and B). This confirms that β -galactosidase-positive cells do not migrate toward the central vein during the life of the animal.

Discussion

Retroviral vectors have already been successfully used to study cell lineage in various organs.^{35,36} This type of vectors allows a stable integration and expression of the marker gene in the progeny of any labeled cell. Moreover, the use of the β -galactosidase gene from *Escherichia coli* coupled to a nuclear localization signal allows a rapid and sensitive histochemical detection of the marker that is concentrated around the nuclear envelope and therefore is clearly distinguishable from any endogenous cytoplasmic β -galactosidase activity. Retroviral infection is limited to dividing cells, and this has long been a limitation of gene transfer to hepatocytes. We have previously shown that following partial hepatectomy, which triggers hepatocytes' entry into the cell cycle, gene transfer to hepatocytes could be achieved *in vivo*.²⁹ The present study demonstrates the feasibility of cell lineage studies in the adult liver during prolonged periods of time.

Two major conclusions can be drawn from the size of the β -galactosidase-positive clusters as well as the spatial distribution of the positive cells in the lobule. The increase in the number of β -galactosidase-positive cells in the clusters with time demonstrates that hepatocytes are able to divide throughout the life of the animal. In the liver of rats analyzed after 15 months, the maximal number of positive cells per clone was approximately 30, indicating that five to six cycles of division had occurred. The first rounds of divisions could have taken place during the regeneration process. Indeed, in livers analyzed 15 days after perfusion, at a time when the regeneration process is completed, we observed the presence of clusters of up to eight cells. This supports the hypothesis that following partial hepatectomy, the duration of the cell cycle of hepatocytes, which is approximately 34 hours, allows them to divide several times before returning to a Go state.^{3,5} The larger clusters in the livers analyzed after 15 months were mainly located in zones 1 and 2. This result is in agreement with a previous study suggesting that the hepatocytes in zone 3 display a lower proliferative capacity.³⁷ Alternatively, we cannot exclude the possibility that all the hepatocytes have the same replicative potential but that apoptosis is predominant around the hepatic vein, thus resulting in a drop in the number of positive cells per cluster observed in zone 3. The presence of isolated positive cells after 15 months could be explained by the absence of any division of some of the labeled cells during the time period of the experiment. Again, we cannot exclude the possibility

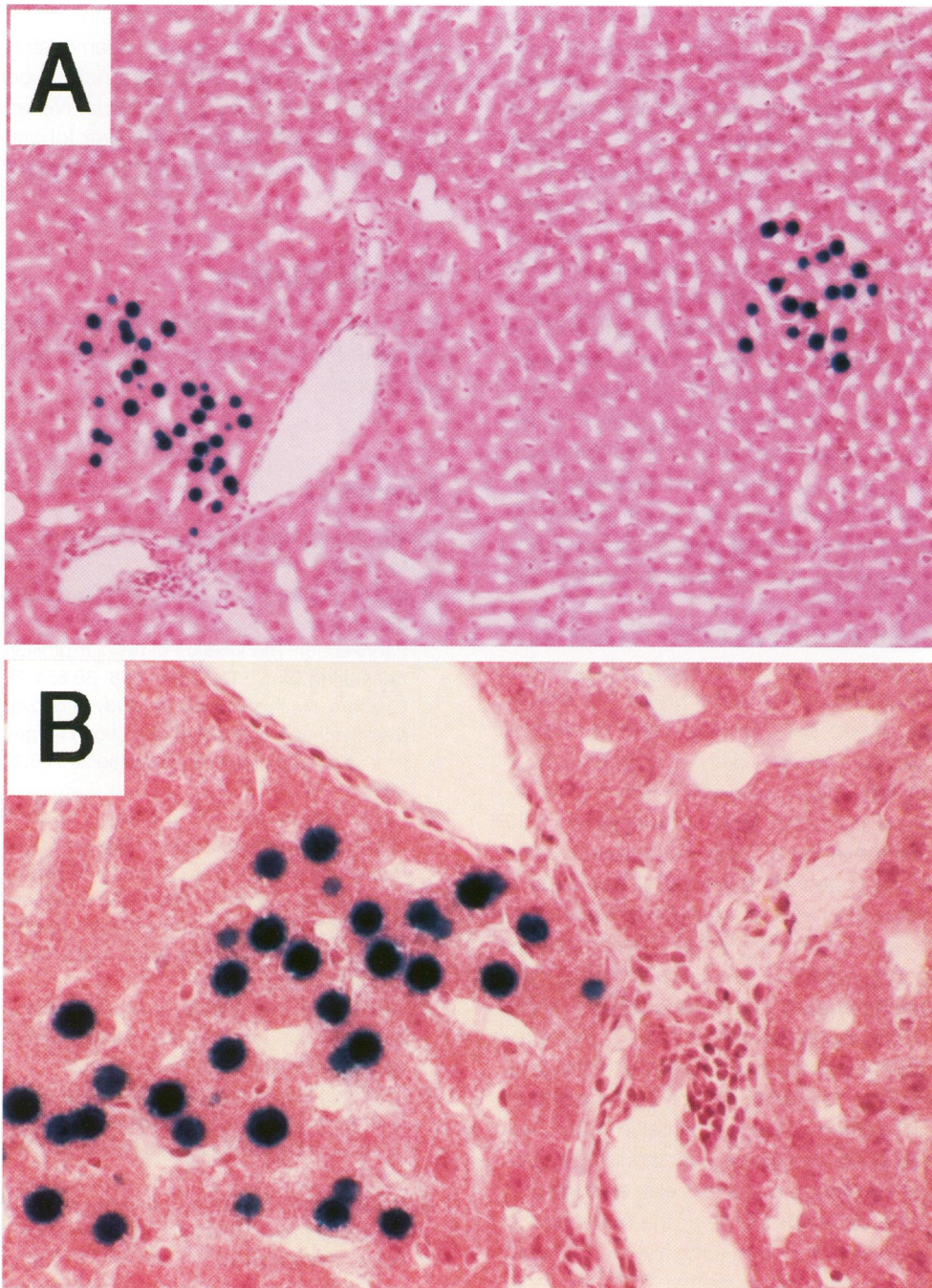


Figure 4. Histochemical detection of β -galactosidase activity in rat liver 15 months after perfusion. Same as Figure 1 except that the animal was sacrificed 15 months after the perfusion. **A:** Presence of two clusters of β -galactosidase-positive cells around a portal space (magnification: 150 \times). **B:** (magnification: 600 \times).

that positive cells in the progeny of labeled hepatocytes may have been eliminated. A single positive cell may therefore be the only remnant witness of the former presence of a small cluster. Altogether, our data are consistent with a life span of hepatocytes between 150 and 450 days, similar to previously estimated values.¹ Positive hepatocytes were always

distributed throughout the liver parenchyma with preferential localization in zones 1 and 2 of the lobule. This confirms that most hepatocytes participate in the regeneration process and that in the first hours after partial hepatectomy the bulk of DNA synthesis is concentrated in zones 1 and 2. The percentage of positive cells in each zone at day 3 (42% in zone 1, 35%

in zone 2, and 23% in zone 3) is in agreement with previous studies of kinetics of DNA synthesis and mitosis in the regenerating liver.³⁻⁵ Indeed, retroviral vectors are able to integrate into cells that will enter the cell cycle within the next 6 hours.^{32,33} Therefore, if the perfusion is carried out between 24 and 27 hours after partial hepatectomy, the population of hepatocytes that enter the cell cycle between 24 and 33 hours will be labeled. In addition, the distribution pattern of the β -galactosidase-positive cells was not modified with time. Fifteen months after perfusion, the proportions of positive clusters in each zone were identical to the values obtained after 3 or 15 days. This clearly demonstrates that there is no apparent or actual migration of hepatocytes in the lobule during regeneration or thereafter. Moreover, the existence of round-shaped clusters of positive cells completely included in zone 1 indicate that the new cells formed by the division of hepatocytes remain in the same zone of the lobule.

In the streaming liver hypothesis, the liver stem cells are thought to divide continuously and give rise to new hepatocytes at the portal rim of the lobule. These newly formed cells are then able to migrate toward the central vein at a daily velocity of 1.43 μ . These results were obtained using [³H]thymidine labeling during a period of time not exceeding 120 days. The change in the distribution pattern of grain counts was interpreted as the result of cell division and displacement as well as variation in the cell fecundity with age. It is known that reutilization of the label may induce artifactual results when using [³H]-thymidine as has already been discussed by some authors.^{26,27} The results obtained in the present report were obtained using genetic labeling of the cells and therefore with no possibility for transmission of the label from cell to cell except by cell division. According to the streaming model, we should have observed β -galactosidase-positive cells arranged in cords lining the sinusoids between the portal space and the central vein. Moreover, such a velocity should have resulted in a journey of more than 650 μ for single positive cells, a distance larger than the size of the lobule, and after 15 months, we should not detect positive cells in zones 1 and 2. On the contrary, our results clearly indicate that hepatocytes are able to divide without moving in the hepatic lobule and therefore argues strongly against the streaming hypothesis. Finally, the presence of clusters in zones 2 and 3 demonstrates that the appearance of the clones is due to hepatocyte division with no participation of putative stem cells that should be present only in the portal space (ie, in the vicinity of zone 1).

In conclusion, our results obtained using *in vivo* genetic labeling of hepatocytes demonstrate that the participation of stem cells does not account for the renewal of hepatocytes in the normal liver and that the diversity of hepatocytes in the liver lobule does not result from a displacement of these cells in the lobule.

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