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Ras-transduced Diethylnitrosamine-treated Hepatocytes Develop into Cancers of Mixed Phenotype *in Vivo*[1]

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ABSTRACT

The cell of origin of hepatocellular carcinoma (HCC) is controversial. A method for marking cells of different lineages *in vivo* and then determining their carcinogenic potential should resolve this issue. A retroviral vector expressing activated *ras* and *β-gal* genes (*Ras-gal*) was transferred into adult rat hepatocytes *in vivo*, and some animals were treated with diethylnitrosamine (DEN). Bile ductule cells and the putative stem cells of the liver (the oval cells) did not appear to be transduced by this method. At 1 month after transfer, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining was performed on transduced rat livers to determine the blue cluster size. Eight % of the clusters in *Ras-gal*-transduced, DEN-treated livers contained at least twice as many cells as the largest cluster in *Ras-gal*-transduced, DEN-untreated rats, demonstrating that they had acquired markedly abnormal growth properties. When the retroviral vector containing *β-gal* without *ras* (Gal-509) was transferred into DEN-treated rats, 2.5% of the cells were present in clusters containing at least twice as many cells as the largest cluster in Gal-509-transduced, DEN-untreated animals. Thus, p21-*ras* may increase the percentage of cells that acquire mutations in response to DEN, or it may behave synergistically with other mutations to increase the replication rate of cells. Occasional foci in *Ras-gal*-transduced, DEN-treated rats had extramedullary hematopoiesis. Forty % of the *Ras-gal*-transduced, DEN-treated rats developed unifocal HCC, mixed HCC/cholangiocarcinoma (CC), or CC at 3–6 months after transduction, suggesting that hepatocytes can develop into HCC or CC if sufficient genetic alterations occur.

INTRODUCTION

HCC[3] is a common tumor worldwide, with an estimated 1 million new cases/year. The development of liver cancer occurs in a stepwise fashion, with frank malignancy resulting once a sufficient number of oncogenes are activated and/or tumor suppressor genes are inactivated (1–4). The overexpression or mutation of *ras* is associated with the development of HCC in spontaneous (5–8), DEN-induced (9–12), aflatoxin-induced (13–15), and transgenic mouse (16, 17) models. p21-ras (18) is a signal transduction protein that is activated in response to a variety of signals including growth factor-ligand binding, and that results in a phosphorylation cascade and activation of genes involved in mitogenesis (19).

Although much is known regarding some of the genes that play a role in the development of liver cancer (1–4), the cellular origin of HCC remains controversial (20–22). Some investigators believe that the hepatocyte can develop into HCC if a sufficient number of mutations accumulate. This theory is supported by the fact that many carcinogenic protocols result in the appearance of foci of hepatocytes with altered gene expression and increased growth properties at intermediate times after the administration of a carcinogenic agent (1, 23). These altered hepatocytes are thought to develop into HCC, although a precursor-product relationship has never been definitively proven. An alternative theory states that putative stem cells of the liver, known as oval cells, undergo developmental arrest and develop into cancer (22, 24). The evidence for this hypothesis stems from the fact that some protocols induce oval cell proliferation without apparent proliferation of hepatocytes at intermediate time points after the administration of a carcinogenic agent. In addition, oval cell proliferation is observed in the intermediate stages of development of cancers in some transgenic mice that express the SV40 T antigen (25, 26).

It has been difficult to distinguish between the above hypotheses because most conditions that predispose to the development of liver tumors induce chronic liver regeneration, leading to replication of oval cells, in addition to hepatocytes. Differentiation between these two theories requires a method to mark cells of known lineage and determine whether they can develop into HCC under the appropriate conditions. Some experiments have been performed by using *ex vivo* genetic modification of cells derived from the liver, followed by implantation of the modified cells into animals (27). RLE cells resemble oval cells in their expression of several histological markers and are thought to be closely related. RLE cells that were genetically modified with an activated *ras* gene resulted in undifferentiated carcinomas that became sarcomatous over time after s.c. implantation into congenic rats (28), or resulted in moderate to well-differentiated HCC upon s.c. implantation into nude mice (29, 30). A similar study reported a variety of different histologies in tumors after s.c. implantation of modified RLE cells. These included undifferentiated tumors, HCCs, and sarcomas; the frequency varied depending on the particular *ras*-transduced RLE cell line used (31). When these same cell lines were implanted into the liver, however, a higher percentage of the tumors were HCC, suggesting that the microenvironment could alter the phenotype of the tumor (32). In still another study, when RLE cells were modified with v-*ras* or v-*raf* genes alone, sarcoma-like tumors resulted upon s.c. implantation into congenic rats (33). In this same study, a vector that expressed both v-*raf* and v-*myc* resulted in RLE cells that developed into HCC *in vivo* (33). Finally, SV40 T antigen-immortalized hepatocytes that were transfected with an activated *ras* gene resulted in poorly differentiated tumors after s.c. implantation in one study (34), and in either moderately differentiated HCC or poorly differentiated HCC in a separate study, depending on the specific clone used (35).

Although the above experiments suggest that both hepatocytes and oval/RLE cells are capable of generating HCC if a sufficient number of mutations accumulate, there are two major drawbacks to these experiments. First, the lineage of the cells isolated after prolonged *in vitro* culture might be incorrect. Although the *ras*-transfected RLE cells expressed α-fetoprotein *in vitro* in one study (30), and the SV40-transformed *ras*-transfected hepatocytes expressed albumin, α_1-antitrypsin, and transferrin (34, 35), it is not clear whether these

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; RLE, rat liver epithelial; DEN, diethylnitrosamine; PH, partial hepatectomy; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PAS, periodic acid-Schiff; PAS-D, PAS with diastase treatment; nt, nucleotide.

genes can clearly differentiate between oval cells and hepatocytes. For example, both oval cells and hepatocytes express albumin and α-fetoprotein (36). Although hepatocytes clearly express α_1-antitrypsin and transferrin, it is not clear whether oval cells express these genes. The second caveat to the above experiments is the variability in results obtained by different investigators using different cells lines and/or clones. This variability raises further questions regarding the lineage of the cells used in different studies. A method for marking cells of a particular lineage *in vivo* and then determining whether they develop into a cancer should help to resolve this issue.

We demonstrated previously that an activated *ras* gene could be delivered to hepatocytes *in vivo* by using a retroviral vector. Activated p21-*ras* resulted in slow replication of hepatocytes, with a doubling time of 3 weeks, as well as loss of glucose 6 phosphatase activity and the accumulation of microvesicular fat.[4] We reasoned that rats whose livers were transduced with *ras in vivo* might develop tumors if a carcinogenic agent was also added. DEN is a DNA-alkylating agent that has been widely used to induce liver cancer in rodents (37). *Ras*-transduced rats were therefore treated with a low dose of DEN, and livers were analyzed for the appearance of cells with abnormal growth characteristics and the development of tumors. The data suggest that activated *ras* may potentiate the appearance of additional mutations that affect the replication rate in response to DEN. In addition, the combination of *ras* and DEN resulted in the appearance of unifocal tumors of mixed phenotype at 3–6 months after transduction. Transduction of liver cells with a retroviral vector expressing an oncogene and treatment with DEN should be a useful method for analyzing the combination of a specific oncogene with a carcinogenic agent.

MATERIALS AND METHODS

Transduction of Rat Liver Cells *in Vivo* and Treatment with DEN. Animal care was in compliance with institutional and NIH guidelines. Male Fisher 344 rats weighing 150–160 gm were obtained from Harlan-Sprague-Dawley and given Purina rat chow and water *ad libitum*. Thirty ml of conditioned medium containing retrovirus was collected from three 15-cm plates of retroviral packaging cells after an overnight incubation at 32°C (38). Polybrene was added to a final concentration of 8 μg/ml, and the retrovirus was injected into the portal vein 24 h after the rats received a 70% PH, as described previously (39). A single batch of conditioned medium was used to transduce 4–6 animals each day on consecutive days until a total of 20 animals had been given injections. One-half of the animals transduced each day were treated with DEN immediately after the injection of retrovirus, which was diluted 1:100 in PBS [139 mM NaCl-2.7 mM KCl-10 mM $NaPO_4$ (pH 7.2)] and administered at 30 mg/kg body weight via gastric gavage (40, 41). The mortality of the transduction procedure was 10% and was not altered by the administration of DEN.

Analysis of Rat Livers. A small wedge biopsy of the liver (<5% of the liver mass) was obtained from the caudate or right lateral lobe during inhaled methoxyflurane anesthesia at various times after transduction. Biopsied animals were followed for the development of tumors. Biopsies were divided, and portions were frozen in OCT in liquid nitrogen for preparation of frozen sections, placed in formalin for preparation of paraffin-embedded slides, or frozen in liquid nitrogen for subsequent preparation of RNA and DNA. Eight-μm frozen sections were stained with X-gal, which results in a blue color in β-gal-expressing cells (42), and counterstained with eosin. Paraffin-embedded sections were stained with hematoxylin and eosin, PAS-D or PAS, or mucicarmine, as described (43). Coded slides were analyzed qualitatively by our pathologist, who was not aware of which vector each animal had received. The diameter of a cluster was determined by using a micrometer. The size of a cluster was calculated by using the formula: volume = $\frac{4}{3}\pi$ radius^3. This volume was used to calculate the number of cells in a cluster, assuming a diameter of 30 μm and a volume of 14,130 μm^3 for each cell. No correction was made for the fact that the diameter of *Ras*-transduced cells was up to ~20% larger than normal hepatocytes. The doubling time was calculated by fitting the data to the equation for first-order exponential growth.

Isolation of RNA. One ml of a solution containing 4 M guanidinium-thiocyanate (Fluka Biochemika, Buchs, Switzerland), 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol was added for each 100 mg of liver tissue. The samples were homogenized with a type B Wheaton dounce, and RNA was isolated as described (44). The amount of RNA was determined by measuring the absorbance at 260 nm. RNA concentration and quality was confirmed by electrophoresis on a formaldehyde-agarose gel and ethidium bromide staining (45).

RNase Protection Assays. *Ras*-SP72 contains the 1.1-kb human K-*Ras* gene (18) cloned into pSP72 (Promega, Madison, WI). It was linearized with *Eco*RI, which cuts 530 nt upstream of the 3′ end of the *Ras* gene, and transcribed with T7 RNA polymerase using [α-^{32}P]GTP at a specific activity of 400 Ci/mmol (46). This resulted in a 550-nt antisense RNA probe with a specific activity of 1×10^8 cpm/μg. Approximately 1×10^5 cpm of probe was incubated with 10 μg of RNA in standard buffers (45), heated to 85°C, slow cooled to 45°C, and hybridized overnight. Samples were digested with 300 μl of digestion buffer containing 1 μg of RNase A (United States Biochemical, Cleveland, OH) and 10 units/ml of RNase T1 (Calbiochem, San Diego, CA) at 30°C for 1 h, extracted with phenol, precipitated with ethanol, and electrophoresed on a 6% polyacrylamide-8 M urea gel with 0.5× TBE (45 mM Tris borate 1 mM EDTA; Ref. 45).

RESULTS

Retroviral Vectors and *in Vivo* Transduction. The retroviral vectors used in this study are shown in Fig. 1. Gal-509 is a control retroviral vector that contains the liver-specific transthyretin promoter and β-gal. *Ras-gal* contains a human Ki-*ras* gene with an activating mutation at amino acid 12 (18), in addition to the β-*gal* gene. The inclusion of the internal ribosome entry site is critical to ensure that a dicistronic mRNA is translated into both proteins in >95% of cells transduced with a retroviral vector (47). Generation of high-titer, replication-incompetent packaging cell lines has been described previously.[4]

Appearance of Large Blue Clusters and Basophilic Foci in *Ras*-transduced, DEN-treated Livers. Rats were transduced *in vivo* at a low multiplicity of infection, and some animals were treated with DEN. Biopsies were obtained at 1 month after transduction, and X-gal staining was performed on liver sections. The expression of β-gal in retroviral-transduced hepatocytes facilitates the identification of transduced cells and the determination of their replication rate by analyzing their cluster size. Fig. 2*A* demonstrates that Gal-509-transduced, DEN-treated rat livers have primarily singlets or doublets of blue

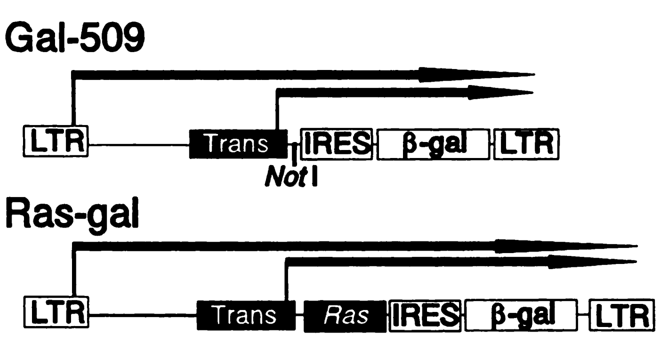

Fig. 1. Retroviral vectors used to transduce rat liver cells *in vivo*. Gal-509 is the control retroviral vector that contains the β-*gal* gene controlled by the liver-specific transthyretin promoter. *Ras-gal* contains a human K-*ras* gene with an activating mutation at codon 12. The encephalomyocarditis internal ribosome entry site (*IRES*) sequence allows a dicistronic mRNA to be translated into two proteins. *Arrows* indicate that transcription can initiate from either the retroviral long terminal repeat (*LTR*) or the internal transthyretin promoter.

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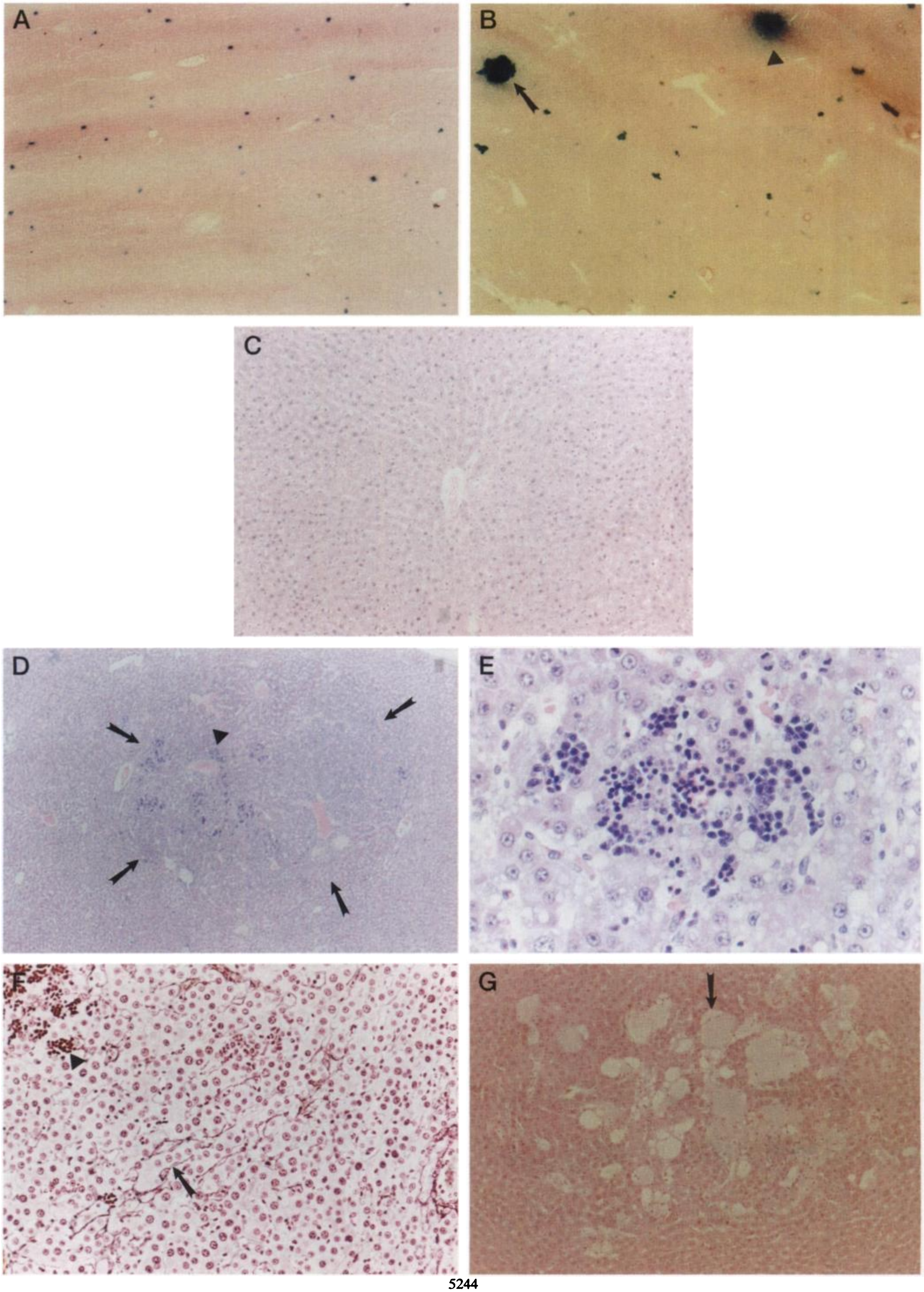
[4] Lin, Y. Z., Brunt, E., Bolling, W., Hafenrichter, D., Flye, M. W., Kennedy, S., and Ponder, K. P. Expression of *ras* from a retroviral vector alters hepatocyte differentiation and replication *in vivo*, submitted for publication.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cells, which demonstrates that little replication had occurred in most transduced cells. In contrast, most of the cells in *Ras-gal*-transduced, DEN-treated rats appeared as small blue clusters (Fig. 2*B*), the size of which was similar to that observed in rats transduced with *Ras-gal* and not treated with DEN (data not shown). This demonstrates that *ras* induced slow replication of most hepatocytes, as expected. Occasional cells appeared as clusters of up to 290 cells, demonstrating that some cells had acquired additional mutations in response to DEN that allowed them to replicate as often as every 3.5 days.

If the large blue clusters observed in *Ras-gal*-transduced, DEN-treated rats are indeed premalignant, they should continue to replicate over time. Unfortunately, no blue cells were observed in most *Ras-gal*-transduced rats at 6 weeks or later after transduction because of down-regulation of β-gal expression in *Ras-gal*-transduced hepatocytes.[4] Rat liver sections from DEN-treated animals were therefore analyzed by hematoxylin and eosin staining at 3 months after transduction because it was previously demonstrated that *Ras*-transduced cells appeared as basophilic foci with this method for up to 10 months after transduction. Fig. 2*C* demonstrates that no basophilic foci were present in a liver section from a Gal-509-transduced, DEN-treated rat. In contrast, basophilic foci of varying size were observed in *Ras-gal*-transduced, DEN-treated rat livers. The focus shown in Fig. 2*D* was calculated to contain at least 1 × 10^5 cells and to have replicated every 5.5 days. This demonstrates that some transduced hepatocytes had continued to replicate at a rapid rate for up to 3 months in *Ras-gal*-transduced, DEN-treated rats. No large foci were observed in *Ras-gal*-transduced rats that were not treated with DEN. Extramedullary hematopoiesis was observed in a small fraction of the foci, as shown in Fig. 2*D* and at higher power in Fig. 2*E*. Although a characteristic feature of HCCs is the absence of reticulin staining, this large focus with markedly abnormal growth properties still maintained a reticulin network, as shown in Fig. 2*F*. Finally, some foci contained cystic degeneration with areas that resembled peliosis at late times after transduction, as shown in Fig. 2*G*.

Quantitative Analysis of Blue Cluster Size in Transduced Rats. An advantage of this *in vivo* retroviral delivery system is the ability to quantify the replication rate of several individual colonies after transduction and treatment with DEN. Fig. 3 demonstrates quantitative analysis of cluster size by X-gal staining at 1 month after transduction for representative animals of each of the 4 experimental groups. For the control vector (Gal-509), all clusters contained 1 or 2 cells from each of 4 animals that were not treated with DEN. For the Gal-509-transduced, DEN-treated rat shown, 98% of the clusters contained 1 or 2 cells, 2% of the clusters contained 6–10 cells, and no larger clusters were observed. For a total of 4 such rats, 2.5% ± 0.55 of the clusters contained 6–10 cells at 1 month after transduction, which was at least twice as many cells as was observed in the largest blue cluster in Gal-509-transduced rats, which were not treated with DEN. This suggests that a small percentage of hepatocytes from animals treated with this low dose of DEN had a slight increase in their replication rate. Over 40% of the hepatocytes from *Ras-gal*-transduced animals that were not treated with DEN were present in clusters of >5 cells, indicating that *Ras* alone induced slow replication, as expected. Eleven % of the clusters from a *Ras-gal*-transduced, DEN-treated rat contained at least twice as many cells as the largest cluster observed in *Ras-gal*-transduced, DEN-untreated rats; the average percentage of clusters containing more than 100 cells was 8 ± 1.4% in four rats. These very large clusters of blue cells probably represent cells that have undergone additional mutations in response to DEN, which allowed more rapid growth to occur.

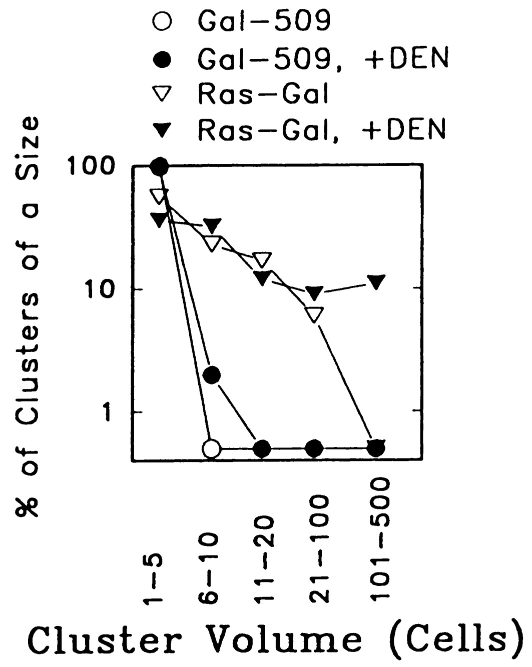


Fig. 3. Quantitative analysis of cluster size at 1 month after transduction. Rat livers were transduced *in vivo* with ~5 × 10^5 colony forming units of Gal-509 or *Ras-gal* and were either treated with DEN or received no further treatment. One month after transduction, liver biopsies were obtained, and sections were stained with X-gal. The diameter of over 50 blue clusters was determined by using a micrometer, and the minimum cluster volume in total cells was calculated as described in "Materials and Methods." The percentage of all clusters containing 1–5 cells, 6–10 cells, 11–20 cells, 21–100 cells, or 100–500 cells is shown for each rat on a semi-log scale.

Appearance of Tumors in Transduced Rats. Animals were followed for 10 months or longer for the appearance of tumors. No tumors developed in rats that received Gal-509 with (n = 9) or without (n = 7) DEN treatment, or in rats that received *Ras-gal* without DEN treatment (n = 7). In contrast, 4 of 10 rats (40%) that were transduced with *Ras-gal* and treated with DEN developed tumors at 3–6 months after transduction. The characteristics of the tumors are summarized in Table 1. Two tumors were primarily HCCs. The gross morphology of one such tumor is shown in Fig. 4*A*. Hematoxylin and eosin staining of this tumor demonstrates a typical HCC with invasion of the blood vessels (Fig. 4*B*). The second tumor,

Fig. 2. (See opposite page.) Analysis of rat livers after *in vivo* transduction with retroviral vectors and treatment with DEN. Rats were transduced *in vivo* with ~5 × 10^5 blue-forming units of control or *ras*-containing retroviral vectors, and DEN was administered by gastric lavage immediately after the injection of retrovirus. Because rat livers contain ~3 × 10^8 hepatocytes after performing a 70% PH, less than 1 retrovirus was delivered for every 500 hepatocytes. *A*, X-gal staining of a rat liver obtained 1 month after transduction with Gal-509 and DEN treatment. *Blue cells* represent transduced cells that are present in singlets or doublets. ×40. *B*, X-gal staining of a rat liver obtained 1 month after transduction with *Ras-gal* and DEN treatment. Most transduced hepatocytes are present in small blue clusters. Occasional cells are present in larger clusters. The cluster identified by the *arrow* and *arrowhead* are 200 and 150 μm in diameter, corresponding to a minimum cluster size of 296 and 126 cells, respectively. This corresponds to a doubling time of 3.5 and 4 days, respectively. ×40. *C*, H&E staining of a rat liver obtained 3 months after transduction with Gal-509 and DEN treatment. No basophilic foci are observed. ×100. *D*, H&E staining of a rat liver obtained 3 months after transduction with *Ras-gal* and DEN treatment. *Arrows*, margins of a large basophilic focus. This focus is 1.5 mm in diameter, which corresponds to approximately 126,000 cells and a doubling time of 5.5 days. *Arrowhead*, region of extramedullary hematopoiesis. ×40. *E*, H&E staining of rat liver obtained 3 months after transduction with *Ras-gal* and DEN treatment. A high-power view of a focus with extramedullary hematopoiesis is shown. ×400. *F*, reticulin staining of a rat liver obtained 3 months after transduction with *Ras-gal* and DEN treatment. A reticulin network (*arrow*) is still present in this large focus, although the plates contain more hepatocytes than usual. *Arrowhead*, extramedullary hematopoiesis. ×200. *G*, H&E staining of a rat liver obtained 10 months after transduction with *Ras-gal* and DEN treatment. Large vascular channels are present within a basophilic focus, as indicated by the *arrow*. ×100.

which was primarily hepatocellular, accumulated microvesicular fat (Fig. 4*C*); this finding was observed in most basophilic foci from *Ras-gal*-transduced rats regardless of whether DEN was administered. Although the pathological characteristics of this tumor was primarily HCC, some cells produced cytoplasmic mucin, as shown in Fig. 4*D*. One tumor had mixed HCC/CC characteristics. PAS staining of the hepatocellular region demonstrated PAS (+) globules within these cells (Fig. 4*E*), as is commonly seen in HCC. Other regions of this same tumor were primarily CC, with glandular formation in a desmoplastic stroma. The presence of mucin was demonstrated by mucicarmine stain (Fig. 4*F*). The last tumor was primarily CC, as demonstrated by hematoxylin and eosin (Fig. 4*G*) and mucicarmine (Fig. 4*H*) staining. Thus, retroviral transduction of hepatocytes with a *ras* oncogene and treatment with DEN resulted in HCC, mixed HCC/CC, or CC.

Tumors from *Ras-gal*-transduced, DEN-treated Rats Express the Human *ras* Genes. None of the 4 tumors that developed in *Ras-gal*-transduced, DEN-treated rats stained blue with X-gal (data not shown). There were 3 potential mechanisms that could explain the absence of blue color after X-gal staining: (*a*) the tumor was transduced with the retroviral vector, and expression still occurred but had attenuated to a level insufficient to stain the cell blue with X-gal; (*b*) the tumor was transduced with the retroviral vector, but expression of β-gal and *ras* had completely shut off; or (*c*) the tumor was never transduced with *Ras-gal* in the first place. To distinguish between these possibilities, RNA was isolated from three of the tumors and analyzed for the presence of human *ras* transcripts by RNase protection assay, as shown in Fig. 5. The presence of human *ras* transcripts demonstrated that these tumors were transduced with the retroviral vector and continued to express the *ras* gene.

DISCUSSION

This study demonstrates that the expression of an activated K-*ras* gene in hepatocytes is synergistic with the carcinogenic agent DEN for the development of liver cancer *in vivo*. In these experiments, the carcinogenic protocol described initially by Scherer and Emmelot (40) and Scherer *et al.* (41) was chosen over other more commonly used regimens because it was most easily adapted to incorporate the retroviral transduction step. Most cancers develop in a stepwise fashion, with frank malignancy resulting once a sufficient number of mutations have accumulated. Although chemical carcinogens play a causal role in the development of liver and other cancers, the interaction between a specific oncogene and a carcinogenic agent has not been studied extensively. In one study, transgenic mice expressing hepatitis B surface antigen, which were treated with aflatoxin B_1 (48) or DNA alkylating agents (49), developed tumors much more rapidly. A possible mechanism for the former synergism is the increased expression of *P450* genes in the hepatitis B surface antigen-expressing mice, leading to increased activation of aflatoxin B_1 to a carcinogenic metabolite (50). Other oncogenes have not yet been analyzed for their ability to synergize with specific carcinogens in the development of liver cancer.

Activated *Ras* May Slightly Increase the Frequency of DEN-induced Mutations That Increase the Replication Rate. The methods described here facilitate the analysis of how individual hepatocytes respond to a carcinogen. Administration of a low dose of DEN to hepatocytes transduced with the control retroviral vector Gal-509 resulted in slow replication of 2.5 ± 0.55% of the cells during the first month after transduction, whereas hepatocytes transduced with Gal-509 and not treated with DEN did not replicate appreciably, as shown

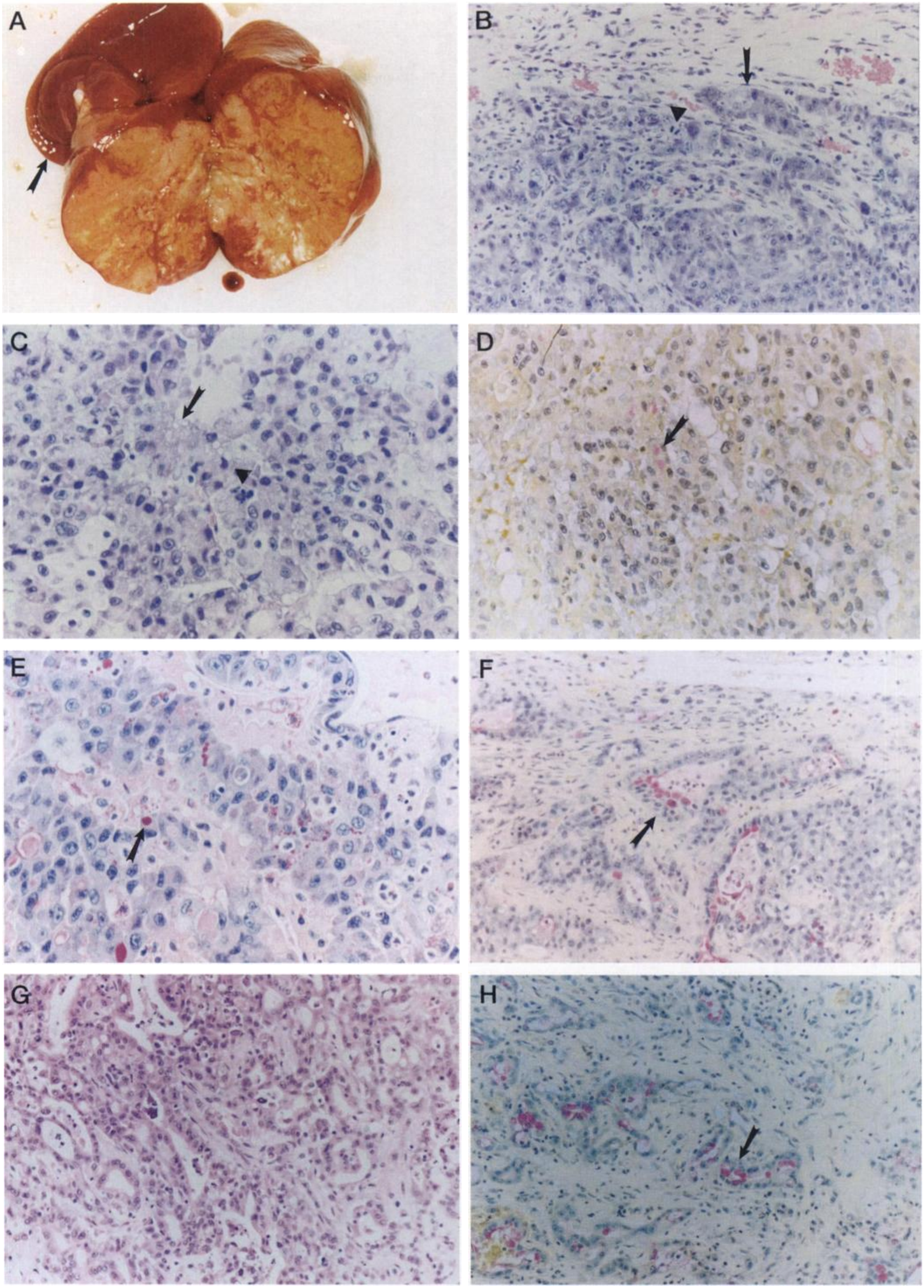
Table 1 *Histopathology of tumors in Ras-gal-transduced, DEN-treated rats*

Four of 10 rats transduced *in vivo* with the Ras-*gal* retroviral vector and treated with DEN developed tumors within 6 months after transduction.

Rat no.	Description of tumor	Tumor dimensions (cm)	Doubling time (days)	Metastases
1566 (3 months)[a]	Moderately differentiated HCC in trabeculae and pseudoglands. Large amounts of tumor necrosis. Eosinophilic PAS-D globules present. Rare cells with intracytoplasmic mucin seen with PAS-D staining. G6Pase (-). Bile (-).	2.5 × 2.5 × 2	3.1	Periadrenal soft tissue
1550 (3.5 months)[a]	Moderately differentiated mixed HCC/CC. Hepatocellular component predominant, with cells in sheets, cords, and trabeculae encased by endothelial cells; some areas cystic. Prominent nucleoli and up to 10 mitoses per high-power field. Intracytoplasmic PAS-D-positive eosinophilic globules in some tumor cells. Microvesicular steatosis in some tumor cells. Reticulin (-), G6Pase (-). Bile absent. CC component glandular with apical staining for mucin with mucicarmine and PAS-D stains. G6Pase (-).	2 × 2 × 2	3.5	Lung and intraabdominal lymph nodes
1546 (3.5 months)[a]	Moderately differentiated HCC, with abundant necrosis and invasion of blood vessels. Rare cells at the periphery with intracytoplasmic mucin after PAS-D staining. G6Pase (-).	5 × 4 × 2.5	3.2	Lung and intraabdominal lymph nodes
1545 (6 months)[a]	CC with large amounts of tumor necrosis and calcifications. Apical and intracytoplasmic mucin demonstrated in the majority of tumor cells with mucicarmine and PAS-D staining. Perineural and intravascular tumor present. G6Pase (-).	3 × 3 × 2.5	6	Lung

[a] Time of onset of the tumor after retroviral transduction.

Fig. 4. (See opposite page.) Gross and microscopic analysis of tumors that result in *Ras-gal*-transduced DEN-treated rats. *A*, gross morphology of the HCC derived from rat 1546. This tumor measured 5 × 4 × 2.5 cm. The tumor has been bivalved. *Bright yellow areas* correspond to necrotic regions within the tumor. *Arrow*, adjacent normal liver. *B*, H&E staining of the HCC from rat 1546. *Arrow*, HCC invading the blood vessels. *Arrowhead*, mitotic figures. ×200. *C*, H&E staining of the HCC from rat 1566. The microvesicular fat accumulation indicated by the *arrow* can be observed in several tumor cells. *Arrowhead*, karryorhetic nucleus. ×400. *D*, mucicarmine staining of the HCC from rat 1566. Although the cells have characteristics of HCC, some cells have accumulated cytoplasmic mucin (*pink*) as indicated by the *arrow*. ×200. *E*, PAS staining of the hepatocellular region of the mixed HCC/CC tumor from rat 1550. The region of the liver tumor from rat 1550 with hepatocellular characteristics contained PAS-positive globules (*purple*) in a number of cells, as indicated by the *arrow*. ×400. *F*, mucicarmine staining of the CC region of the mixed tumor from rat 1550. Apical mucin (*pink*) is present in glandular regions of the tumor, as indicated by the *arrow*. ×200. *G*, H&E staining of the CC from rat 1545. Glandular structures are present in a desmoplastic stroma. ×200. *H*, mucicarmine staining of the CC from rat 1545. The glandular structures, identified by *arrows*, have accumulated mucin, as evidenced by their *pink* color. ×200.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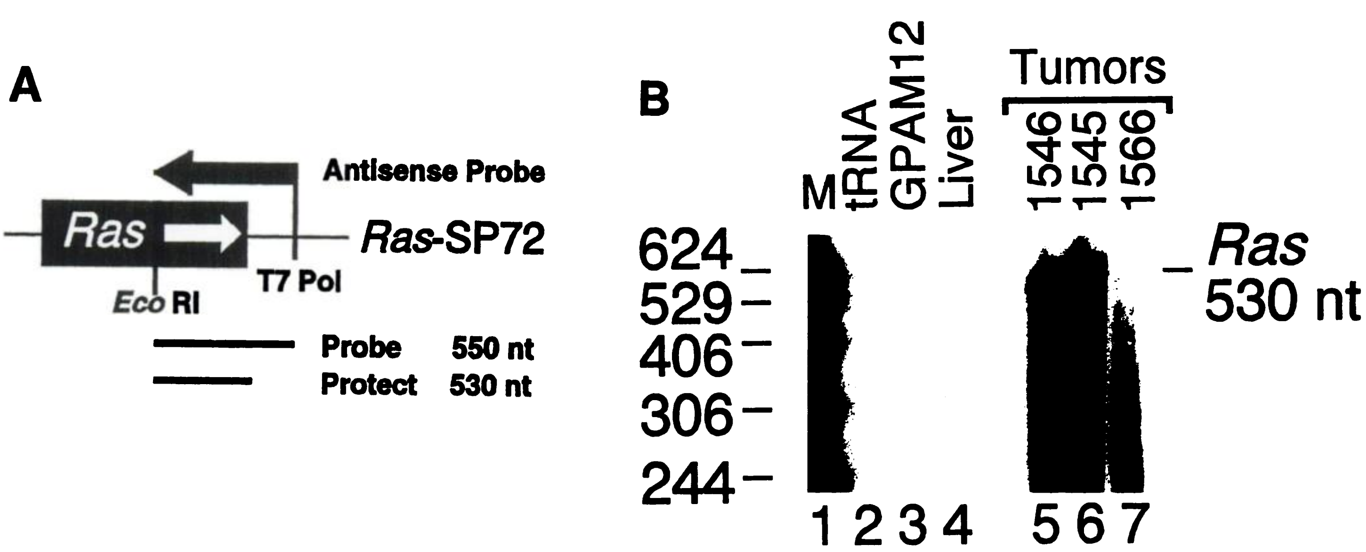

Fig. 5. *Ras* mRNA is expressed in tumors from *Ras-gal*-transduced, DEN-treated rats. *A*, schematic diagram of the RNA probe used in the RNase protection assay. A radiolabelled antisense RNA of 550 nt was synthesized *in vitro* by T7 RNA polymerase (*T7 pol*). After incubation with a sample containing *ras* RNA, a 530-nt RNA is protected from single-strand specific RNases. *B*, RNase protection assay using the *ras* probe. Ten μg of sample RNA were hybridized with the antisense *ras* probe shown in *A*, digested with single strand specific RNases, and electrophoresed on a 6% denaturing polyacrylamide gel. *Lane 1* (*M*), end-labeled *Msp*I-digested pBR322, with the size of DNA markers in nt shown on the *left*. *Lane 2*, RNA from yeast (*tRNA*); *Lane 3*, RNA from nontransfected GP + AM12 packaging cells (*GPAM12*); *Lane 4*, RNA from nontransduced rat liver (*Liver*). RNA from tumor 1546 (*Lane 5*), 1545 (*Lane 6*), and 1566 (*Lane 7*) clearly contained *ras* RNA, as evidenced by the signal at 530 nt. The signal in *Lane 7* is of lower molecular weight because the RNA obtained from that tumor was partially degraded.

in Fig. 3. Analysis of blue cluster size from Gal-509-transduced rats at later time points demonstrated a similar frequency of larger blue clusters in DEN-treated rats as compared with DEN-untreated rats, although the number of cells in the larger clusters increased over time (data not shown). This frequency of apparent mutation in response to DEN and PH is considerably higher than what has been reported after the administration of a similar dose of DEN in previous studies. For example, abnormal growth appeared in less than 1 of 3000 cells when ATPase-negative (40, 41) or placental glutathione *S*-transferase-positive (51) foci were analyzed. This discrepancy is probably due to the fact that all foci in the previous studies had alterations in gene expression in addition to abnormal growth properties, whereas this study only required that an abnormal growth rate be present. Thus, a major advantage of the retroviral marking studies reported here is the ability to visualize individual liver cells and assess their replication rate simply by observing their cluster size.

The methods described here may facilitate the analysis of oncogene-carcinogen interactions in the early stages of liver cancer. An oncogenic mutation might be synergistic with a carcinogen by inducing division of a cell before its DNA damage can be repaired. Alternatively, an oncogenic mutation may have no effect on the incidence of additional mutations in response to carcinogens but will simply increase the probability of developing cancer by serving as the first step in a multistep process. Eight % ± 1.4% of clusters in *Ras-gal*-transduced, DEN-treated rats had at least twice as many cells as the largest cluster in *Ras-gal*-transduced, DEN-untreated rats, whereas only 2.5 ± 0.55% of the blue clusters from Gal-509-transduced, DEN-treated hepatocytes had twice as many cells as the largest cluster in Gal-509-transduced, DEN-untreated hepatocytes. This indicates that activated p21-*ras* may potentiate the mutagenic effect of DEN up to 3-fold. The following caveats should be recognized, however: (*a*) the background replication rate in rats that did not receive DEN was markedly higher for the *Ras-gal*-transduced hepatocytes as compared with the Gal-509-transduced cells, complicating a direct comparison of the percentage of cells that acquired additional mutations by the analysis of cluster size; (*b*) some mutations may act synergistically with *ras* to further increase the replication rate but have no effect on the replication rate of cells when they occurred alone. Thus, activated *ras* may have had a slight effect on the rate at which additional mutations occurred in response to a carcinogen, but more experiments with other oncogenes and/or tumor suppressor genes will be necessary to confirm this finding.

Some Foci in *Ras-gal*-transduced, DEN-treated Rats Support Extramedullary Hematopoiesis. A small fraction of the basophilic foci that developed in *Ras-gal*-transduced, DEN-treated rats supported extramedullary hematopoiesis. Extramedullary hematopoiesis was not observed in any livers from *Ras-gal*-transduced rats that did not receive DEN, or in any Gal-509-transduced rats regardless of DEN treatment. Sandgren *et al.* (16) also noted foci of basophilic hepatocytes that supported extramedullary hematopoiesis at 2 weeks after birth in transgenic mice expressing low levels of *ras* from the albumin promoter/enhancer. Thus, the data presented here, as well as previous data, suggest that activated *ras* in conjunction with additional mutation(s) can create an environment conducive to seeding and replication of hematopoietic cells. Although the liver is the major site of hematopoiesis in the fetus, this process shifts to the bone marrow after birth. Conditions that result in hematopoiesis of one or more lineages in the adult rodent liver include induction of hepatocyte replication by PH (52, 53), systemic administration of interleukin 3 (54), or induction of severe anemia by administration of phenylhydrazine (55). In addition, oval cells produce stem cell factor (56), whereas cultured hepatocytes can produce erythropoietin or granulocyte-macrophage colony-stimulating factor (57). We hypothesize that hepatocytes that express activated *ras* and some additional factor(s) may be induced to express hematopoietic growth factors that result in seeding and replication of hematopoietic cells in the liver. Experiments are currently under way to determine whether these foci specifically express hematopoietic growth factors, as compared with other foci that do not support hematopoiesis.

The Hepatocyte Is a Source for HCC and CC. The data presented here suggest that hepatocytes transduced with a retroviral vector containing an activated *ras* gene can serve as a source for HCC and CC. There are three pieces of data that led to the conclusion that the parenchymal hepatocyte is the only cell type modified with the retroviral vector: (*a*) hepatocytes are the only cells that stained blue with X-gal after *in vivo* delivery of retroviral vectors containing the transthyretin (Fig. 2), the cytomegalovirus early promoter (58), or the long terminal repeat promoter (59) to the liver. In addition, retroviral vectors containing the phosphoglycerol kinase promoter or the promoter for the large subunit of RNA polymerase II upstream of the *β-gal* gene only resulted in blue hepatocytes after *in vivo* transduction and X-gal staining;[5] (*b*) retroviral transduction only occurs in replicating cells (60). Because the retrovirus was injected at 24 h after PH and other cells in the liver are not replicating at this time (61), it is logical that the hepatocyte should be the only cell type modified. Furthermore, oval cells are not present in appreciable numbers in the normal rat liver (62); and (*c*) no proliferation of bile ductules was observed at early times after transduction. In contrast, Sandgren *et al.* (16) observed bile ductule proliferation at 1 day after birth in transgenic mice given injections of an albumin promoter/enhancer upstream of an activated *ras* gene. Because the albumin gene is expressed in oval cells but not in bile ductule cells (36), their results suggest that expression of *ras* in oval cells should lead to bile ductule proliferation in animals. The absence of bile duct proliferation in our study suggests that oval cells were not transduced, although it is possible that their proliferative response might be blunted in adults.

Three pieces of data demonstrate that the tumors that arose in *Ras-gal*-transduced DEN-treated rats contain the retroviral vector and expressed its genes: (*a*) a RNase protection assay performed on RNA isolated from the tumors resulted in full-length protection of the human *ras* probe (Fig. 5), which was complementary to 530 nt of the 3′-coding and 3′-untranslated sequence of human K-*ras* mRNA. Because the human (63) and the rat (64) K-*ras* coding sequence is <90% conserved, and because RNase protection assays result in cleavage at single nt mismatches (65), the rat K-*ras* RNA should not result in full-length protection in this assay. Furthermore, β-gal RNA was found in the tumor samples by RNase protection assay (data not shown). Because the *β-gal* and *ras* genes are present in the transduced cells on a dicistronic RNA, this provides further evidence that the human *ras* gene is expressed in the tumors; (*b*) Southern blot analysis with a restriction enzyme that cuts once within the retroviral vector demonstrated that all tumors contained a single copy of the provirus integrated into a unique site (data not shown); and (*c*) DEN treatment induced tumors in rats transduced with *Ras-gal* but not in rats transduced with Gal-509. This requirement for *ras* for the development of tumors at early time points is consistent with previous data demonstrating that tumors do not develop until at least 14 months after PH and administration of DEN at the dose used in this study (40, 41).

Thus, the parenchymal hepatocyte is the only cell type that appeared to be modified with the retroviral vector in these experiments, and the tumors that developed were clearly transduced by the retroviral vector. This suggests that the hepatocyte is the source of both the HCC and CC tumors that occurred. This result is consistent with previous data demonstrating that SV40-immortalized, *ras*-transfected hepatocytes could develop into tumors *in vivo* (34, 35). Although these data support the theory that hepatocytes can develop into HCC, they do not argue against the possibility that earlier progenitor cells such as oval cells can also be responsible. Indeed, although these two theories are often presented as mutually exclusive hypotheses, it is likely that either hepatocytes or oval cells can develop into tumors with some characteristics of liver cells if they acquire a sufficient number of mutations.

The appearance of tumors of mixed HCC and CC phenotype in human patients (66) and in experimentally induced cancers (14) is consistent with other data that suggests that a cell with the potential to develop along both the hepatic and the biliary lineage exists. The expression of markers of more than one lineage is not unique to the liver because hematopoietic tumors occasionally express markers of two or more different hematopoietic lineages (67). Uriel (23) suggested that differentiated cells can "retrodifferentiate" into a more immature progenitor cell in response to an oncogenic stimulus, and that these cells can then recapitulate their developmental potential and develop into tumors with regions containing characteristics of two or more different lineages (23). The fact that one mixed HCC/CC tumor and one CC tumor developed in this study after transduction of hepatocytes with the *ras* oncogene supports the hypothesis this hepatocytes can retrodifferentiate into cells capable of differentiating along the biliary pathway.

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