Alterations in Enzymatic Functions in Hepatocytes and Hepatocellular Carcinomas From *Ras*-Transduced Livers Resemble the Effects of Insulin

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An understanding of how oncogenes affect differentiated liver functions might lead to improved treatments for liver cancer or other disorders where liver-specific functions are compromised. A retroviral vector that coexpressed β-galactosidase (β-gal) and activated *Ras* genes (*Ras-gal*) was transduced into a small fraction of adult rat hepatocytes *in vivo*. Hepatocytes from *Ras-gal*–transduced diethylnitrosamine-untreated livers and hepatocellular carcinomas (HCC) from *Ras-gal*–transduced diethylnitrosamine-treated rats were analyzed for liver functions by performing histochemical assays on liver sections. *Ras-gal*–transduced hepatocytes failed to express gluconeogenic, ketogenic, and urea pathway enzymes. In contrast, several enzymes involved in fat synthesis were strongly activated, and microvesicular fat accumulated. These metabolic changes are induced in normal livers by insulin, a hormone that activates *p21-ras*. The deregulation of *p21-ras* may inhibit these liver-specific functions and may induce fat synthesis in both malignant and nonmalignant liver diseases. Furthermore, treatment with drugs that inhibit the attachment of *p21-ras* to the plasma membrane might reverse these changes. The alterations in enzymatic functions in the HCCs were similar to those observed in the hepatocytes, although each of the two cancers had a region that abruptly lost its expression of liver-specific enzymes and acquired the expression of genes that are more characteristic of oval or bile ductule cells. This suggests that a single genetic event in a malignant cell may dramatically alter its apparent phenotype. The identification of this putative gene might lead to insights into the regulation of the phenotype of normal cells in the liver. (HEPATOLOGY 1996;24:838-848.)

Abbreviations: HCC, hepatocellular carcinoma; GK, glucokinase; HK, hexokinase; TGF-α, transforming growth factor α; β-gal, β-galactosidase; DEN, diethylnitrosamine; cDNA, complementary DNA; HE, hematoxylin-eosin; SD, succinate dehydrogenase; β-HBD, β-hydroxybutyrate dehydrogenase; UDPGD, uridine diphosphogluconate dehydrogenase; G6PD, glucose 6-phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; 6PGD, 6-phophogluconate dehydrogenase; NADH, reduced form of nicotinamide adenine dinucleotide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; GS, glycogen synthase; G6Pase, glucose 6-phosphatase; 5′NT, 5′ nucleotidase; ALP, alkaline phosphatase; OCT, ornithine carbamoyl transferase; G3PD, glyceraldehyde 3-phosphate dehydrogenase; IRES, internal ribosome entry site; α-GPD, α-glycerophosphate dehydrogenase; γGT, α-glutamyl transpeptidase; LDH, lactate dehydrogenase.

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Neoplastic transformation of the liver is caused by the activation of oncogenes and to the inactivation of tumor suppressor genes.[1-4] A feature of both normal and malignant cells is the inverse correlation between their replication rates and degrees of differentiation.[5-8] Understanding how oncogenes can affect differentiated functions might lead to improved treatments for both malignant and nonmalignant liver diseases for several reasons. First, this information might lead to pharmacological treatments that may restore differentiation and hence inhibit the replication of malignant cells. The most striking example of this strategy is found in acute promyelocytic leukemia, where retinoic acid promotes the differentiation of and inhibits the replication of leukemic cells in human patients *in vivo*.[9] This strategy may also have implications for liver cancer. Rat liver epithelial cells are similar to oval cells, which are putative pluripotent liver stem cells. Although malignant rat liver epithelial cells resulted in aggressive cancers after subcutaneous implantation, they differentiated into hepatocytes after implantation into the liver, and no cancers developed.[10] Thus, the induction of differentiation of an otherwise malignant liver-derived cell prevented it from replicating. Second, the identification of oncogene-activated genes might lead to liver cancer–specific therapies. For example, some cancers, including hepatocellular carcinomas (HCC), have increased levels of glucokinase (GK), which is the high K_m hexokinase (HK) that phosphorylates glucose.[11] A GK inhibitor, mannoheptulose, inhibited the glucose uptake and the growth of tumor cell lines *in vitro* and *in vivo*.[11] Third, oncogenes might be activated in nonmalignant conditions, such as in hepatitis or cirrhosis. If that occurs, the knowledge of how specific oncogenes affect differentiated functions might enable one to devise pharmacological strategies that restore liver functions in these disease states.

p21-ras is activated when insulin or growth factors, such as transforming growth factor α (TGF-α), bind to their cognate receptors on the outside of cells.[12,13] Activated *p21-ras* activates downstream signalling molecules, such as mitogen activated protein kinase, which phosphorylates transcription factors such as *Fos* and *Jun* family members.[12] The overexpression or mutation of *Ras* genes is common in liver cancers in rodents.[1-4,14-20] Although some investigators failed to identify *Ras* mutations in HCC in humans,[21] others have reported *Ras* mutations in human HCC[22-24] or in a human hepatoma cell line.[25] Thus, *Ras* genes play an important role in the acquisition of the malignant state in liver-derived cells.

We previously described a retroviral vector that coexpressed β-galactosidase (β-gal) and an activated *K-ras* gene from a liver-specific promoter (*Ras-gal*).[26] Although *Ras-gal* alone resulted in the replication of hepatocytes, no cancers were observed for up to 10 months after transduction. When *Ras-gal* was delivered to the hepatocytes of rats that were treated with a low dose of the liver carcinogen diethylnitrosamine (DEN), however, liver cancers developed at 3 to 6

months after transduction in 40% of the animals.[26] The purpose of this study was to determine the effect of activated *p21-ras* upon differentiated liver functions in hepatocytes from *Ras-gal*–transduced DEN-untreated rats, and in liver cancers from *Ras-gal*–transduced DEN-treated rats. We demonstrate here that *p21-ras* by itself results in the inhibition of enzymes that are involved in gluconeogenesis, ketogenesis, and urea formation, and in the activation of genes that are involved in fat synthesis. Thus, *p21-ras* reproduces some of the effects of insulin upon liver metabolism and may contribute to the liver insufficiency that is observed in some patients with HCC. Enzymatic functions were also analyzed in two HCC that developed in the *Ras-gal*–transduced DEN-treated rats. Although most of both cancers resembled the hepatocytes from the *Ras-gal*–transduced DEN-untreated livers, both had a region with an abrupt loss of liver-specific functions and an activation of oval cell/bile ductule functions. This suggests that a single genetic event that occurs upon a background of other mutations may result in the loss of hepatic functions and in the activation of functions that are characteristic of cells of an earlier or related lineage. The identification of this putative differentiation factor might lead to insights into normal liver development.

MATERIALS AND METHODS

Generation of Retroviral Vectors. *pRS-8391-BN* is a Moloney murine leukemia virus-based retroviral vector with a unique *Not I* site that is immediately downstream of the unique *Bgl II* site in *pRS-8391.*[27] The *IRES*-*dihydrofolate reductase gene cassette and part of the *3′ LTR* were removed from *pRS-8391-BN* by *Xba I* restriction and were replaced with a 4.4 kb *Xba I* fragment containing *IRES-β-gal*[28] and the same portion of the *3′ LTR* in order to generate *pBN-IRES-gal.* The 360-bp transthyretin enhancer/promoter[29] was cloned into the *Bgl II* site upstream of the *IRES* of *pBN-IRES-gal* to generate *Gal-509. Ras-gal* was generated by inserting a 1.1-kb fragment of a human *K-ras2* complementary DNA (cDNA) with a point mutation at *codon 12*[30] into the *Not I* site of *Gal-509.* Each vector was cotransfected with *PGK-neo*[31] into the amphotropic retroviral packaging cell line *GP + AM12,*[32] and neomycin-resistant colonies were selected with 0.5 μg/mL of *G418.* A conditioned medium from colonies was used to infect *NIH 3T3* cells,[33] as described previously,[27] and the number of blue forming units (bfu) after staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (*X-gal*) was determined.[34]

Focus Formation on Soft Agar. *NIH 3T3* cells were infected with conditioned medium from retroviral packaging cells with 8 μg/mL of polybrene at a multiplicity of infection of 0.1. The day after infection, 10,000 cells were plated onto a 6-cm plate in 0.3% agar.[30] Plates were evaluated 3 weeks later for the presence of transformed foci. The average result of duplicate infections ± SD is reported.

***Transduction of Rat Hepatocytes* In Vivo.** The animals received care that was in compliance with institutional and National Institutes of Health guidelines. Male Fisher 344 rats weighing ~ 160 g (Harlan Sprague Dawley, Indianapolis, IN) were given Purina rat chow (Ralston Purina, St. Louis, MO) and water *ad libitum.* For retroviral transduction, 5 mL of a conditioned medium containing 8 μg/mL of polybrene and $\sim 5 \times 10^5$ bfu of retroviral vector was injected into the portal vein 24 hours after the rats had received a partial hepatectomy of 70%, as described previously.[27,34] Some animals were also treated with a single dose of 30 mg/kg of DEN, administered by gavage, immediately after an injection of the retrovirus. Biopsy samples were obtained from the rats whose food had been withheld for 20 hours during anesthesia with inhaled methoxyflurane. Portions of each biopsy were frozen in optimal cutting temperature compound (Bayer Corp., Mishawaka, IN) or placed in formalin for the preparation of paraffin-embedded slides.

Analysis of Liver Sections. The paraffin-embedded sections were stained with hematoxylin-eosin (HE) or with periodic acid-Schiff as described.[35] Coded slides were analyzed qualitatively by our pathologist. Eight-micrometer frozen sections were stained with *X-gal* after fixation with 1.25% glutaraldehyde for 10 minutes on ice[27] or were stained for fat with Oil Red O.[35] Other enzymatic reactions were performed on unfixed sections, unless otherwise stated, and were postfixed for 20 minutes in a 4% formaldehyde, 2% calcium chloride 7.5% polyvinyl-pyrrolidone (average molecular weight, 40 kd) solution. Unless otherwise stated, all reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Simple Dehydrogenase Reactions. The following reactions involved the addition of a substrate that could directly undergo a dehydrogenase reaction, resulting in the reduction of nitroblue tetrazolium to a blue-violet product. They were performed according to slight modifications of the previously published protocols in a solution containing 50 mmol/L Tris(hydroxymethyl)amino-methane [pH 7.4]), 20% polyvinyl alcohol (average molecular weight, 30-70 kd), 5 mmol/L nitroblue tetrazolium, 5 mmol/L magnesium chloride, 0.32 mmol/L phenazine methosulfate, and 10 mmol/L sodium azide. The succinate dehydrogenase (SD) reaction[36] was performed on sections that were fixed for 30 seconds in 1.25% glutaraldehyde in cold phosphate-buffered saline at 4°C and used 50 mmol/L succinate. For this reaction, the polyvinyl alcohol was omitted, and the Tris was replaced by 50 mmol/L sodium phosphate buffer (pH 7.5). The following dehydrogenase reactions contained 1 mmol/L nicotinamide adenine dinucleotide and 10 mmol/L β-hydroxybutyrate (β-HBD),[37] 25 mmol/L α-glycerophosphate (α-GPD),[38] 5 mmol/L uridine diphosphogluconate (UDPGD),[39] or 50 mmol/L lactate dehydrogenase (LDH).[38] For the glutamate dehydrogenase reaction, 7.5 mmol/L nicotinamide adenine dinucleotide and 100 mmol/L glutamate[40] were used, 50 mmol/L sodium phosphate buffer (pH 7.8) replaced the Tris buffer, and the reaction was performed under argon. Other dehydrogenase reactions contained 0.8 mmol/L nicotinamide adenine dinucleotide phosphate and 10 mmol/L glucose 6-phosphate (G6PD),[36] 5 mmol/L isocitrate (ICDH),[41] 11 mmol/L 6-phophogluconate (6PGD),[36] or 10 mmol/L malate.[36]

Other Simple Reactions. The reduced form of nicotinamide adenine dinucleotide (NADH) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) reductase assays[37] used (3-[4,5 dimethylthiazol-2]-2,5-diphenyltetrazolium bromide and previously published methods. Glycogen phosphorylase[42] and glycogen synthase (GS)[39] were assayed for their ability to convert D-glucose-1-phosphate or uridine diphosphate -glucose into glycogen, respectively. Lipase[40] was assayed for its ability to release fatty acids from either Tween 60 (a mixture of 16 and 18 carbon acyl chains) or Tween 20 (12 carbon acyl chains). The following reactions released phosphate, which was detected according to modifications of the method of Gomori.[43] Glucose 6-phosphatase (G6Pase),[44] 5′ nucleotidase (5′NT),[40] alkaline phosphatase (ALP),[40] ATPase,[45] ornithine carbamoyl transferase (OCT),[46] adenylate cyclase,[47] and γ-glutamyl transpeptidase (γGT)[48] were performed exactly as described by others.

Multi-Step Reactions. Some of the reactions required sequential enzymatic steps. For these assays, the appropriate substrate was added in addition to any upstream or downstream enzymes that were necessary to produce a histochemically visible product. GK and HK reactions[49] were performed with 100 mmol/L and 0.5 mmol/L glucose, respectively, using the conditions described for the G6PD reaction with 5 mmol/L adenosine triphosphate, 0.5 mmol/L nicotinamide adenine dinucleotide, and exogenous G6PD at 1 U/mL. The phosphofructokinase reaction[42] included 26 mmol/L fructose 6-phosphate and exogenous downstream enzymes (yeast aldolase and glyceraldehyde 3-phosphate dehydrogenase [G3PD]) at 1 U/mL. The aldolase reaction[42] included fructose 1,6 diphosphate (F16DP) at 12 mmol/L and the exogenous downstream enzyme G3PD at 1 U/mL. Both phosphofructokinase and aldolase reactions were assayed, as described previously, with 0.32 mmol/L phenazine methosulfate and 10 mmol/L sodium azide. For the G3PD reaction,[50] 9 mmol/L F16DP was added in the presence of the exogenous upstream enzyme aldolase at 1 U/mL because G3P is very unstable. Pyruvate kinase was assayed as described previously[51] with exogenous HK and G6PD at 1 U/mL, except 20% polyvinyl alcohol was used instead of a semipermeable membrane to reduce the diffusion of reaction products.

RESULTS

Production of Retroviral Vectors. The control retroviral vector *Gal-509,* shown in Fig. 1, contained the liver-specific transthyretin promoter upstream of the internal ribosome entry site (*IRES*)-*β-gal* cassette. To create *Ras-gal,* a *K-ras2* gene with a point mutation at *codon 12* was placed downstream of the transthyretin promoter in *Gal-509.* This mutation results in a constitutively active *Ras* protein that can transform cells. The *IRES* binds to the ribosome, leading to the translation of a downstream gene in a cap-independent fashion. Dicistronic messenger RNAs containing the *IRES*

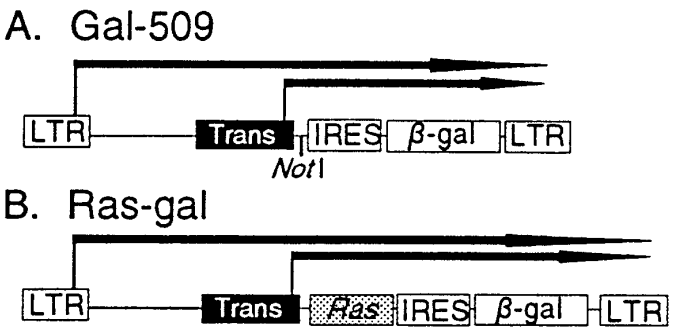


FIG. 1. Retroviral vectors. (A) Control retroviral vector *Gal-509.* The long-terminal repeats (LTRs) are necessary to produce a retroviral vector. The liver-specific transthyretin promoter (Trans) is expressed long-term from a retroviral vector in the liver. The encephalomyocarditis internal ribosome entry site sequence (IRES) allows the downstream gene of a dicistronic mRNA to be translated into a protein. The *β-gal* gene encodes an enzyme that allows expressing cells to be visualized by *X-gal* staining. The arrows indicate that transcription can initiate from either the LTR or the transthyretin promoter; either transcript can be translated into both proteins. (B) Retroviral vector *Ras-gal.* This construct is identical to *Gal-509,* except that a *K-ras* gene with a point mutation at *codon 12,* which results in a constitutively activated protein, has been inserted downstream of the transthyretin promoter and upstream of the *IRES–β-gal* cassette.

were previously shown to result in the coexpression of both genes in >95% of cells.[28] Retroviral vectors were transfected into an amphotropic packaging cell line, and high-titer clones were selected. *Gal-509* and *Ras-gal* had titers of 1×10^5 and 8×10^4 bfu/mL, respectively. These retroviral vectors were shown to be free of wild-type retrovirus by performing a marker-rescue assay.[52] To demonstrate the biological activity of the activated *K-ras* gene, retroviral-infected *NIH 3T3* cells were tested for focus formation on soft agar. Uninfected *NIH 3T3* cells had 11 foci per plate. *Ras-gal*–transduced cells had 484 ± 125 foci per plate, which was significantly different from the 17 ± 4.2 foci that were observed on plates from *Gal-509*–transduced cells ($P = .03$). This shows that the activated *ras* gene was biologically active from the *Ras-gal* retroviral vector.

X-gal *and G6Pase Activity in Retroviral-Transduced Rat Livers.* Retroviral particles were delivered to rat livers *in vivo,* using a technique in which the hepatocyte is transduced.[27,34] Liver biopsy specimens were obtained at various times after transduction, and *X-gal* staining was performed on the frozen sections to identify the transduced cells. Both *Gal-509* and *Ras-gal* resulted in singlets and doublets of blue cells, dispersed randomly throughout the liver at 1 week after transduction, while livers from rats that had been transduced with a retroviral vector that contained an irrelevant gene had no blue cells (data not shown). At 1 month after transduction, the blue cells from *Gal-509*–transduced livers remained as singlets, doublets, or very small clusters (Fig. 2A). The minimal-cluster volume was determined by using the formula: volume = 4/3 π radius^3. The minimal number of cells in a cluster was determined by assuming a hepatocyte diameter of 30 μm and a volume of 14,130 μm^3/cell. For 400 blue clusters from 4 separate *Gal-509*–transduced rats, the average cluster volume was 1.6 ± 0.4 cells (± SEM) at 1 month after transduction. The cluster size remained small at 5 months after transduction, as shown in Fig. 2B, with an average cluster volume of 2.4 ± 0.9 cells. This indicates that little replication occurred in the hepatocytes that were transduced with the control retroviral vector. Figure 2C shows that all blue cells from *Gal-509*–transduced rat livers had normal G6Pase activity. This indicates that retroviral transduction and the expression of *β-gal per se* did not interfere with this liver-specific function. All of the blue cells from several *Gal-509*–transduced DEN-untreated livers maintained G6Pase activity at 1 to 10 months after transduction.

In contrast, *Ras-gal*–transduced hepatocytes were in blue clusters with 9.5 ± 1.1 cells/cluster in biopsy specimens that were obtained at 1 month after transduction, as shown in Fig. 2D. Thus, the majority of transduced cells replicated, suggesting that *p21-ras* levels were sufficient to exert a biological effect. The adjacent sections, shown in Fig. 2E and 2F, show two blue clusters that lost G6Pase activity. Similar results were obtained in *Ras-gal*–transduced DEN-treated rat livers, in which all blue foci were negative for G6Pase (Fig. 2G and 2H). The evaluation of numerous adjacent sections from 10 different rats at 1 to 7 weeks after transduction with *Ras-gal* showed that all blue clusters (>1,000 were examined) were negative for G6Pase activity, and that all G6Pase-negative foci were blue, regardless of DEN treatment. We thus consider the loss of G6Pase activity to be a sensitive and reliable marker for *Ras-gal*–transduced cells. Surprisingly, some G6Pase-negative foci from *Ras-gal*–transduced rat livers failed to stain blue with X-gal at later times after the transduction. This is shown in Fig. 2I and 2J for a liver from a DEN-untreated rat that was obtained 7 months after transduction. We believe that these foci represent *Ras-gal*–transduced cells that express activated *p21-ras* at levels that are sufficient enough to inhibit G6Pase activity. We believe that these cells do not stain blue because the level of expression from the retroviral vector had decreased, resulting in levels of *β-gal* that were insufficient to result in blue cells after *X-gal* staining, as will be discussed below.

Formalin-fixed paraffin-embedded sections from *Ras-gal*–transduced and *Gal-509*–transduced livers were also analyzed. *Gal-509*–transduced DEN-untreated rat livers appeared normal after HE staining as early as 1 week to as late as 10 months after transduction, with no altered foci seen (Fig. 3A). In contrast, all *Ras-gal*–transduced DEN-untreated rats had foci of basophilic, enlarged hepatocytes that were randomly dispersed throughout the acinus in livers that were analyzed between 1 and 10 months after transduction. Because the blue clusters observed after *X-gal* staining of a frozen section were basophilic after HE staining of an adjacent frozen section (data not shown), we believe that the basophilic foci observed on formalin-fixed samples represent *Ras-gal*–transduced cells. Figure 3B shows two basophilic foci from a *Ras-gal*–transduced DEN-untreated liver at 2 months after transduction. The higher-power view, shown in Fig. 3C, shows that one of these basophilic foci contained intracytoplasmic vesicles that are consistent with microvesicular steatosis within some of the cells, a finding that was observed in approximately one third of the basophilic foci at 2 months after transduction. The microvesicular steatosis in *Ras-gal*–transduced DEN-untreated rat livers was even more dramatic at 5 months after transduction (see Fig. 3D), when >75% of the basophilic foci contained some cells with microvesicular fat. To definitively prove that these vesicles contained fat, Oil Red O staining was performed. Figure 3E

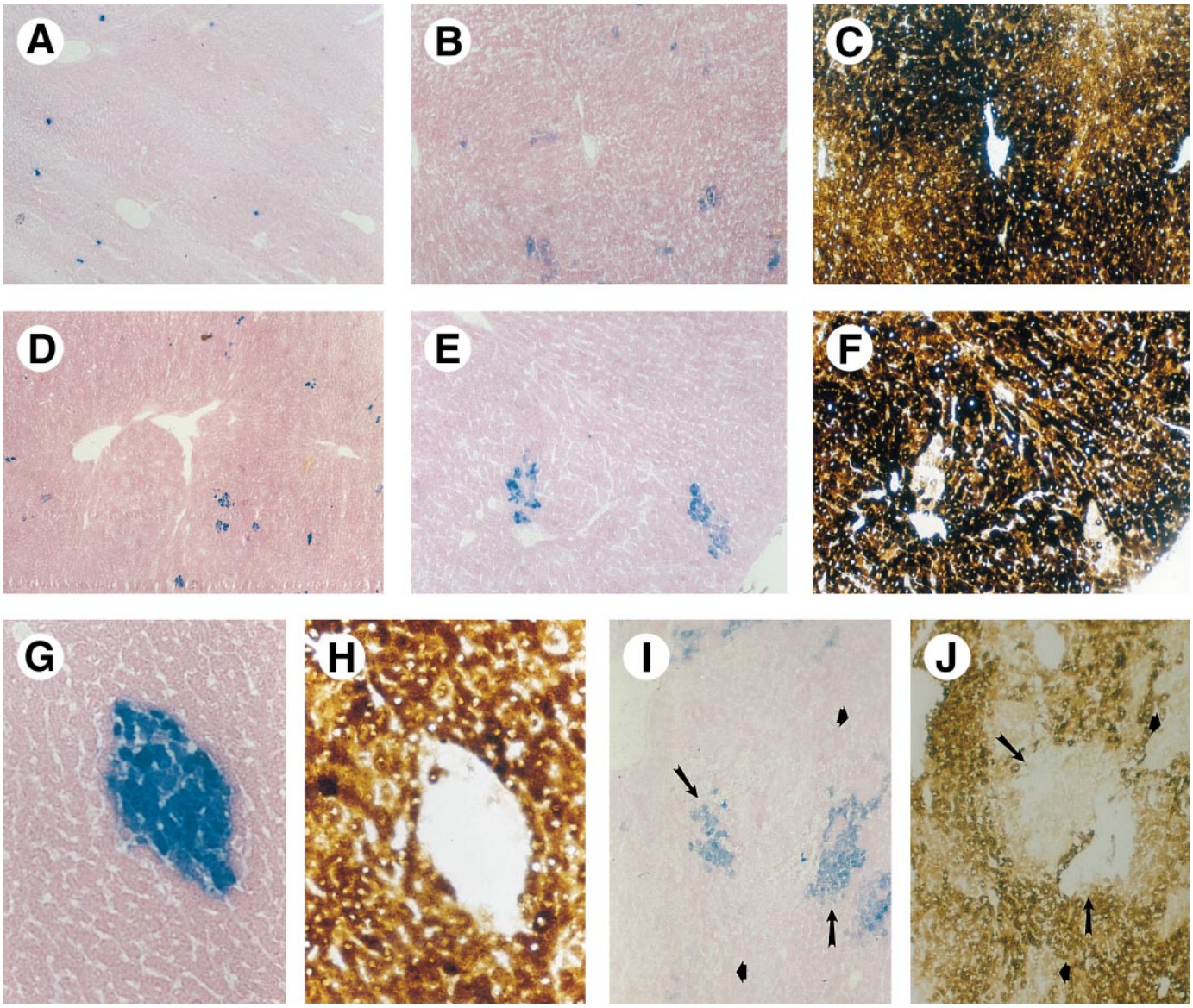


FIG. 2. *Ras-gal* results in the increased replication of hepatocytes and loss of G6Pase activity. Rats were transduced *in vivo* with $\sim 5 \times 10^5$ bfu of control or *ras*-containing retroviral vectors. Because rat livers contain $\sim 3 \times 10^8$ hepatocytes after performing a partial hepatectomy of 70%, <1 retrovirus was delivered for every 500 hepatocytes. Liver biopsy specimens were obtained at various times after transduction and stained for *β-gal* or G6Pase activity. (A-C.) Livers from *Gal-509*–transduced DEN-untreated rats. (A) Shows a liver section from a DEN-untreated rat at 1 month after transduction with *Gal-509* that was stained with *X-gal* and counterstained with eosin. Blue cells remain as singlets or doublets, showing that little replication has occurred. (×40) (B) Shows a liver section from a DEN-untreated rat at 5 months after transduction with *Gal-509* that was stained with *X-gal* (×100) Blue cells remain as singlets, doublets, or small clusters demonstrating that minimal replication has occurred. (C) Shows a section adjacent to that shown in panel B that was stained with G6Pase. All transduced cells have normal G6Pase activity, demonstrating that retroviral transduction and *β-gal* activity *per se* do not interfere with the G6Pase assay. (×100) (D-J) Livers from rats transduced with *Ras-gal* (D) (×40) and (E) (×100) show *X-gal* staining of a liver from a DEN-untreated rat at 5 weeks after transduction with *Ras-gal.* The blue cells are present in small clusters, demonstrating that they have replicated. (F) G6Pase staining of a section adjacent to that shown in panel E. Both blue clusters have clearly lost G6Pase activity. (×100) (G) *X-gal* staining of a *Ras-gal*–transduced DEN-treated rat liver obtained 1 month after transduction. (×100) The blue cluster is larger than any that were observed from *Ras-gal*–transduced DEN-untreated livers of rats, suggesting that the DEN induced additional mutations that further increased the replication rate. (H) Shows a G6Pase staining of a section adjacent to that shown in panel G. This focus lost G6Pase activity. (×100) (I) shows *X-gal* staining of a *Ras-gal*–transduced DEN-untreated liver of a rat at 7 months after transduction, whereas (J) shows G6Pase staining of an adjacent section. (×100) The long, narrow arrows indicate two foci that stained blue with *X-gal* and have a complete absence of G6Pase. The short arrows identify two G6Pase-negative foci that did not stain blue with *X-gal.* (×100)

shows large red globules within cells of a large focus, indicating that the vesicles contain fat. In contrast, the vesicles did not stain with periodic acid-Schiff (see Fig. 3F), showing that they did not contain glycogen. Thus, activated *p21-ras* altered the fat metabolism in many but not all cells from the basophilic foci that were observed in *Ras-gal*–transduced DEN-untreated livers. No cells from *Gal-509*–transduced rats accumulated microvesicular fat at any point in time. At all time points, apoptotic figures were rare, suggesting that *p21-ras* did not induce programmed cell death.

Histochemical Assays in* Ras-gal–*Transduced Hepatocytes and HCC. The major purpose of this study was to define the liver functions that were altered by activated *p21-ras.* Rat livers were isolated at various time points, and frozen liver sections were evaluated histochemically. Because *p21-ras* is activated by insulin, the rats were starved for 20 hours before isolating their livers to decrease the levels of endogenous insulin. Enzymatic assays were performed on liver sections from *Ras-gal*–transduced DEN-untreated rats at 2, 5, 7, and 10 months after transduction, and from *Gal-509*–transduced

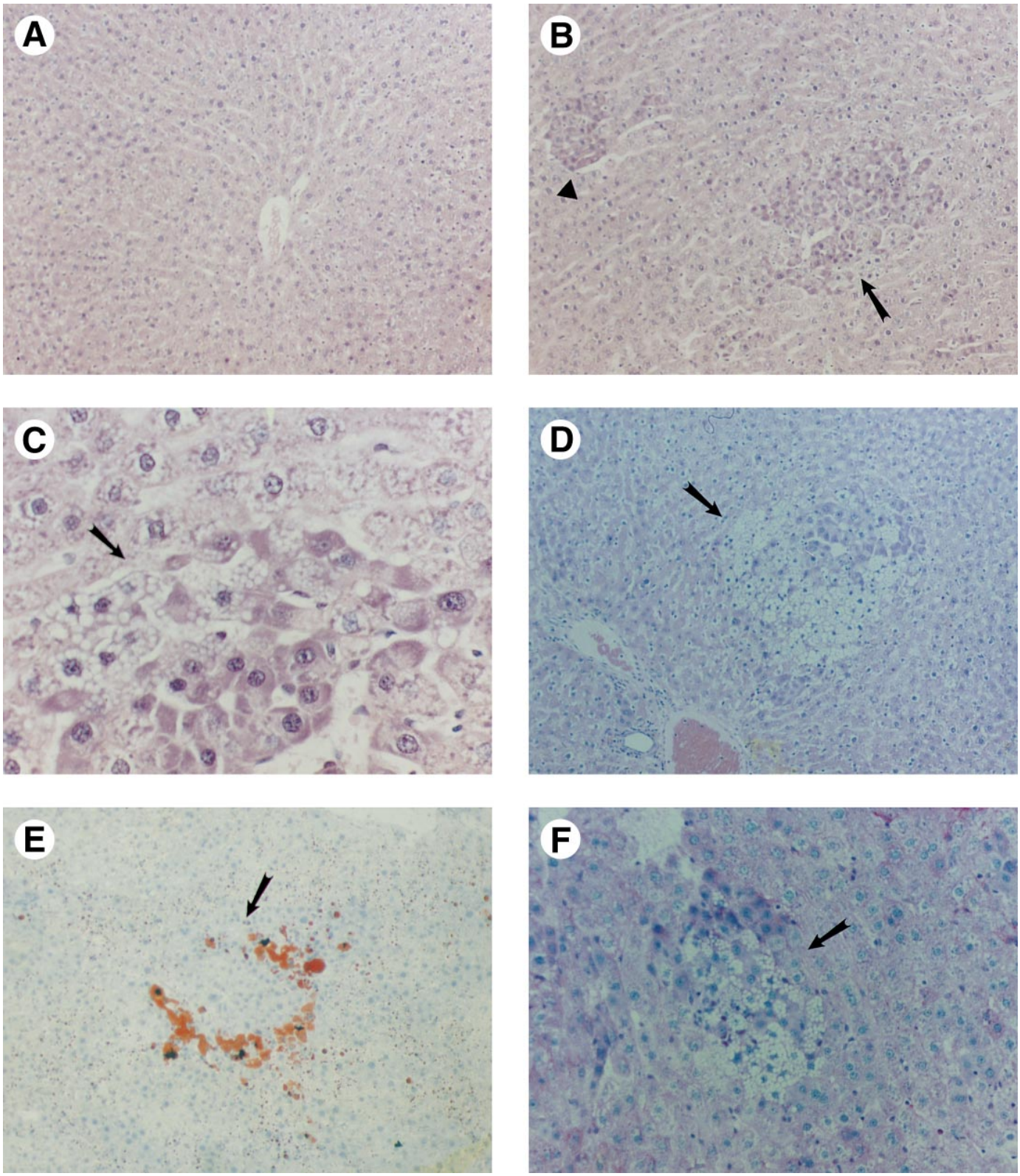


FIG. 3. *Ras*-transduced hepatocytes from livers of DEN-untreated rats appear as basophilic foci with microvesicles that contain fat but not glycogen. Rats were transduced with *Gal-509* or *Ras-gal,* as described in Fig. 2, and the livers from DEN-untreated animals were harvested at various times after transduction. (A) *Gal-509*–transduced DEN-untreated rat liver. This shows HE staining of a section from a liver that was harvested 2 months after transduction with *Gal-509.* No basophilic foci are observed (×100). (B and C) HE staining of a liver obtained 2 months after transduction with *Ras-gal* from the liver of a DEN-untreated rat. Two basophilic foci are indicated in panel B (×100). The long arrow identifies a basophilic focus that contains multiple small vesicles within some of the cells, as shown at a higher power in panel C (×400). (D) HE staining of a liver obtained 5 months after transduction with *Ras-gal* from the liver of DEN-untreated rat. Most of the cells present in the basophilic focus, identified by the arrow, contain large amounts of small vesicles. (×100) (E) Oil red O staining of a *Ras-gal*–transduced liver from the liver of a DEN-untreated rat at 7 months after transduction. The arrow indicates several red vesicles that contain fat. (×100) (F) PAS without diastase staining of a liver isolated 2 months after *Ras-gal* transduction from the liver of a DEN-untreated rat. The vesicles in this focus are negative, showing that they do not contain glycogen (×100).

DEN-untreated rats at 5 and 10 months after transduction. In addition, two of the previously reported HCC from *Ras-gal*–transduced DEN-treated rats[26] were analyzed. *β-Gal*–expressing cells from *Gal-509*–transduced rats were identified by *X-gal* staining and had normal activity for all liver enzymes that were tested on adjacent sections (data not shown). This shows that retroviral transduction and the expression of *β-gal* do not interfere with the histochemical assays. G6Pase was used to identify *Ras-gal*–transduced foci because it is a less expensive and a more sensitive indicator of *Ras-gal*–transduced cells than is *X-gal* staining, as discussed below. The expression of several liver enzymes was altered in *Ras-gal*–transduced hepatocytes. With one exception, 100% of 10 to 20 G6Pase-negative foci were activated, had no change, or were inactivated for the other enzymatic activity. The only exception was ALP, for which ~50% of the G6Pase-negative foci were activated.

Results of Enzyme Assays in* Ras-gal–*Transduced DEN-Untreated Hepatocytes. Several enzymes were inactivated in hepatocytes from DEN-untreated rats by activated *p21-ras.* These include OCT (Fig. 4A and 4B), NADPH-reductase (Fig. 4C and 4D), β-HBD (Fig. 4E and 4F), and canalicular ATPase (Fig. 4G and 4H). In addition, NADH-reductase was decreased, as summarized in Table 1. The expression of other enzymes was not altered in the hepatocytes that expressed activated *p21-ras.* These include SD (Fig. 4I and 4J) and G3PD (Fig. 4K and 4L). In addition, glycogen phosphorylase, GS, HK, aldolase, pyruvate kinate, lipase, adenylate cyclase, and glutamate dehydrogenase were not affected in *Ras-gal*–transduced hepatocytes (see Table 1). ICDH (Fig. 4M and 4N), G6PD (Fig. 4O and 4P), and 5′NT (Fig. 4Q and 4R) were strongly activated in hepatocytes that were transduced with *Ras-gal.* In addition, GK, phosphofructokinase, malate dehydrogenase, α-GPD, LDH and UDPGD activities were increased by activated *p21-ras* (Table 1). ALP was weakly activated in ~50% of the G6Pase-negative foci. Finally, all *Ras-gal*–transduced foci were negative for γGT.

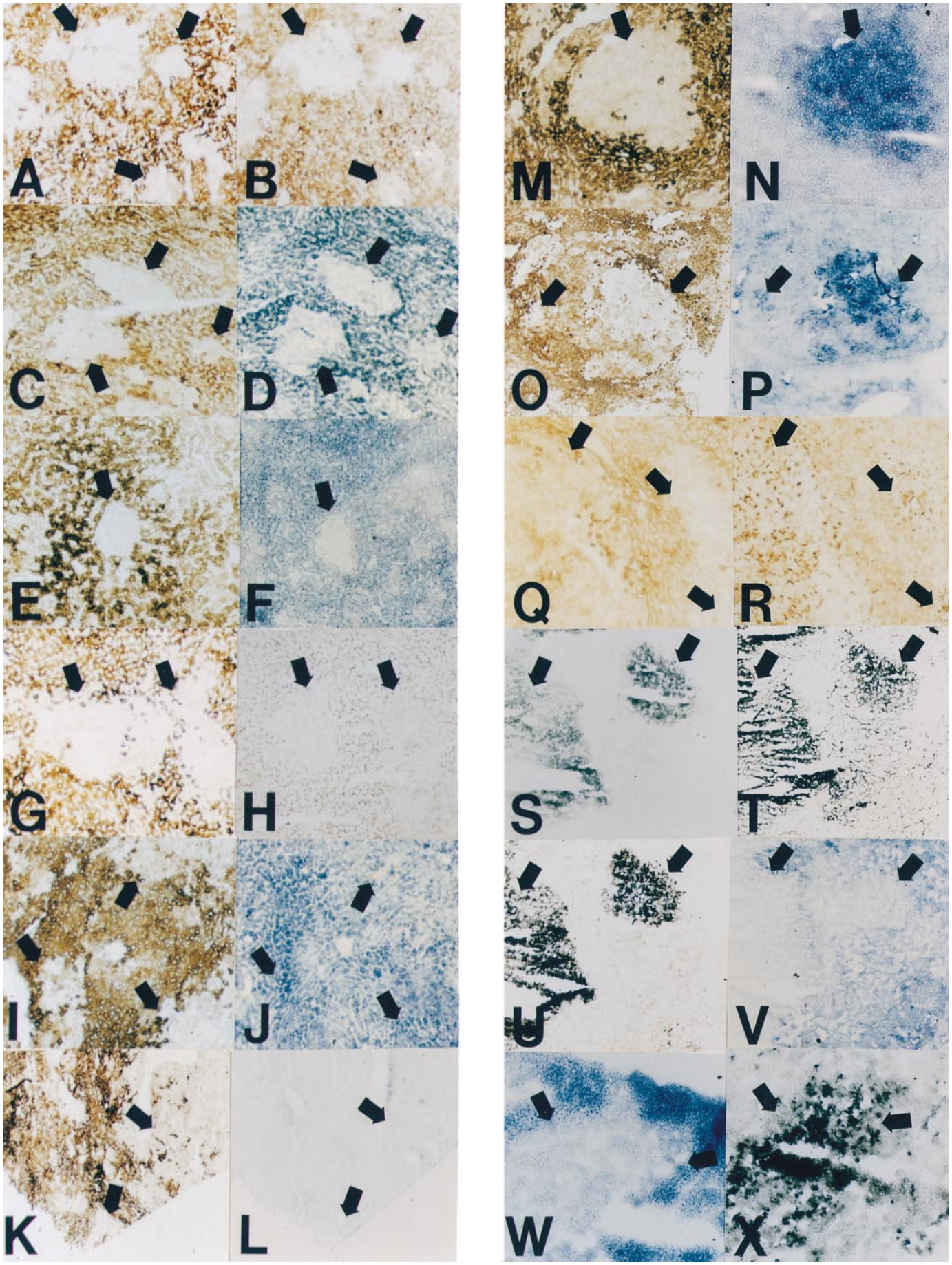
Enzymatic Assays in HCC from* Ras-gal-*Transduced DEN-Treated rats. The above studies examined the liver functions in *Ras-gal*–transduced DEN-untreated hepatocytes. They, therefore, represent foci that probably have only a single genetic change, the expression of activated *p21-ras.* For cancers to develop, several mutations must accrue in a cell which might further alter liver gene expression. We used the same histochemical methods to analyze two of the HCC that developed in *Ras-gal*–transduced DEN-treated rats. Both of these cancers were described previously as moderately differentiated HCC, although *cancer 1546* contained a small region with intracytoplasmic mucin.[26] In this study, each of the HCCs had 2 regions. Region A resembled the *Ras-gal*–transduced foci from DEN-untreated animals, whereas region B was dramatically different. For *cancer 1546,* region B had a strong activation of genes that are more characteristic of bile ductule cells such as γGT (Fig. 4S) and ALP (Fig. 4T). In addition, lipase was strongly activated (Fig. 4U), although it is unclear what enzymatic activity this represents, and whether or not this is a marker for a particular lineage. Intriguingly, region B of *cancer 1546* abruptly lost the expression of several enzymes that are normally expressed at high levels in the liver, such as GK (Fig. 4V), GS, G3PD, ICDH, malate dehydrogenase, G6PD, α-GPD, adenylate cyclase, LDH, and UDPGD (Table 1). This suggests that a single additional genetic change in the malignant cells may have inhibited the hepatic functions and activated the functions characteristic of cells of an earlier or related lineage. Similarly, region B of *cancer 1566* lost the expression of hepatic functions such as ICDH (Fig. 4W), malate dehydrogenase, G6PD, 6PGD, α-GPD, and LDH (Table 1), and had a strong activation of ALP (Fig. 4X). Region B of *cancer 1566* did not, however, activate γGT (Table 1).

DISCUSSION

The effects of oncogenes upon differentiated liver functions is relevant to liver disease for three reasons. First, the identification of the genes that are activated in HCC might lead to therapeutic targets. Second, the identification of mechanisms by which oncogenes lead to dedifferentiation might enable one to restore the differentiation of hepatoma cells and thus inhibit their replication rate. Third, oncogenes might effect liver-specific functions in nonmalignant liver diseases, because proto-oncogenes are present in normal livers and play a role in metabolic signalling pathways. In this report, we have transferred a retroviral vector containing an activated *K-ras* gene into rat liver *in vivo* and have determined the effect upon a number of differentiated functions in both hepatocytes and neoplastic livers. We demonstrate that activated *p21-ras* can affect a large number of liver functions, many of which are induced by insulin. In addition, we demonstrate that the two HCC that developed in *Ras-gal*–transduced DEN-treated rats had a region with an abrupt loss of hepatic functions and an activation of functions that are more characteristic of cells of the oval cell/bile ductule lineage. This suggests that a single genetic event can dramatically alter the apparent lineage of a cell.

Features of the* Ras-gal *Retroviral Vector and the Experimental Design. The retroviral vector *Ras-gal* was designed to coexpress activated *ras* and *β-gal* genes from a dicistronic messenger RNA. Although dicistronic messenger RNA with an *IRES* have resulted in the coexpression of two proteins,[28] the success of this experiment depends on the premise that cells that stain blue with *X-gal* do indeed express activated *p21-ras. Ras-gal* clearly encoded a functional oncogene, since it transformed *NIH 3T3* cells *in vitro.* Furthermore, *Ras-gal* led to liver cancers in 40% of the rats that received a low dose of DEN, although no cancers developed in the *Gal-509*–transduced rats that received an identical experimental procedure and a dose of DEN.[26] Finally, the average blue cluster volume in the *Ras-gal*–transduced rat livers at 1 month after transduction was 9.5 ± 1.1 cells, while the average blue cluster volume in the *Gal-509*–transduced rat livers was only 1.6 ± .4 cells ($P < .0001$). This strongly suggests that activated *p21-ras* expression was sufficient to induce the replication of most hepatocytes, which is consistent with its known biological effects. We therefore conclude that *p21-ras* was expressed from the retroviral vector at biologically significant levels in rat livers.

A second feature of the experimental design relates to the use of G6Pase staining to identify *Ras-gal*–transduced hepatocytes. At 1 month after transduction, all of the >1,000 blue clusters in *Ras-gal*–transduced rats, but none of the >400 blue clusters from *Gal-509*–transduced rats, had lost G6Pase staining. In addition, all of the sections from the *Ras-gal*–transduced DEN-untreated livers, obtained from different rats at 1, 2, 3, 4, 5, 7, or 10 months after transduction, had several G6Pase-negative foci, while no G6Pase-negative foci were seen in the sections from *Gal-509*–transduced DEN-untreated rats at the same timepoints. We therefore consider the loss of G6Pase staining to be a sensitive and reliable indicator of *Ras-gal* transduction. G6Pase staining was used to identify *Ras-gal*–transduced cells in subsequent experiments for 2 reasons: (1) the G6Pase assay is cheaper to perform, and hundreds of slides were analyzed in this study; and (2) G6Pase staining was a more sensitive indicator of *Ras-gal*–transduced cells than was *X-gal* staining, as some G6Pase-negative foci from *Ras-gal*–transduced rats were not blue after *X-gal* staining at the late timepoints. We believe that all G6Pase-negative foci express both activated *p21-ras* and *β-gal,* but that the *β-gal* levels were insufficient to result in detectable blue cells after *X-gal* staining in some foci.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There are other data that suggest that *X-gal* staining is not a sensitive assay. A cell must express >1,000 molecules of *β-gal* to result in blue cells after *X-gal* staining.[53] Indeed, the cancers that developed in *Ras-gal*–transduced DEN-treated rats did not stain blue with *X-gal,* although they expressed *ras* and *β-gal* RNA, had retroviral DNA sequences, and had detectable *β-gal* activity in an *o*-nitrophenyl β-D-galactopyranoside assay,[53] performed on liver extracts.[26] Finally, several lines of transgenic mice that contained the human α_1-antitrypsin promoter upstream of *β-gal*[54] had detectable *o*-nitrophenyl β-D-galactopyranoside activity in liver extracts yet failed to stain blue with *X-gal* (Ponder K, unpublished data). Thus, *X-gal* staining may fail to identify cells that are clearly expressing *β-gal* by other criteria.

Activated* p21-ras *Inhibits Gluconeogenesis, Ketogenesis, and Urea Formation. Activated *p21-ras* resulted in the loss of G6Pase, β-HBD, and OCT activity in DEN-untreated rats. These enzymes contribute to gluconeogenesis, ketogenesis, and urea formation, respectively.[55] These results suggest that the activation of *p21-ras* is sufficient to induce these metabolic changes. It is unclear if other enzymes of these pathways are inhibited by *p21-ras,* as this study was limited to the enzymes for which a simple histochemical assay was available. The loss of G6Pase in *Ras-gal*–transduced cells is consistent with the loss of G6Pase in TGF-α–expressing transgenic mouse hepatocytes,[56] as TGF-α results in the activation of *p21-ras* upon binding to the epidermal growth factor receptor.[12-13] Our results are also consistent with the fact that 14 different transgenic mouse founders that expressed *Ras* at high levels in liver cells died in neonatal life.[57] We hypothesize that these animals were deficient in some critical liver function.

Liver insufficiency can occur in patients with hepatoma, leading to hypoglycemia and hyperammonemia due to the inability to perform gluconeogenesis and to synthesize urea, respectively.[58] Our results suggest that the activation of *p21-ras* might play a role in the liver insufficiency that develops in patients with HCC or nonmalignant liver disorders. We are currently determining if liver insufficiency in animals can be ameliorated by 3-hydroxy-3-methylglutaryl-coenzyme A reductase or by farnesyltransferase inhibitors. These drugs inhibit the production of farnesyl or its transfer to proteins, respectively, and also inhibit the function of *p21-ras* by preventing it from associating with the plasma membrane.[59-61]

p21-ras *Activates Some Enzymes Involved in Fat Synthesis.* The striking accumulation of microvesicular fat in the basophilic foci from the livers of *Ras-gal*–transduced DEN-untreated rats was probably partially because of an increase in fat synthesis. Two enzymes of the pentose phosphate shunt, G6PD and 6PGD, and an enzyme that is required for glycerol synthesis, α-GPD, were strongly activated by *p21-ras.* The pentose shunt is critical for the generation of NADPH for the synthesis of fatty acids, while glycerol forms the backbone for triglycerides. GK, which phosphorylates glucose to produce the nondiffusible G6P that is necessary for fat synthesis, was also activated in *Ras-gal*–transduced hepatocytes. Thus, several enzymes that are important for fat synthesis were activated in *Ras-gal*–transduced hepatocytes. It is unclear if the fat degradation was altered; although lipase was not altered in the *Ras-gal*–transduced cells, the identity of the enzyme whose activity was tested in the histochemical assay is uncertain. Because Tween compounds are monoesters, the lipase activity that was analyzed here is probably not a triglyceride lipase.[62] We are currently analyzing the RNA from *Ras-gal*–transduced and control livers to determine if the expression of other genes that are involved in fat synthesis and degradation are altered.

Pathological fat accumulation occurs in nonmalignant liver disorders such as diabetes mellitus, alcoholic liver disease, viral hepatitis, and acute fatty liver deposits of pregnancy.[62] It is possible that the activation of *p21-ras* plays a role in these disorders, and that drugs that prevent the farnesylation of *p21-ras* might prevent fat accumulation.

p21-ras *Does Not Mimic All of the Effects of Insulin/Feeding Upon Hepatocytes* In Vivo. The loss of gluconeogenesis, ketogenesis, urea formation, and activation of fat synthesis are induced by insulin, a hormone that signals in part by the activation of *p21-ras.*[63] Thus, our data suggest that *p21-ras* is sufficient to induce these changes, and that other insulin-activated signalling pathways are not required. In contrast, other enzymes, such as aldolase, pyruvate kinase, GS, and G3PD, that are activated by insulin and/or by feeding were not affected in the *Ras-gal*–transduced foci. Our results are consistent with the known requirement for glucose in addition to insulin for inducing aldolase and pyruvate kinase in cultured hepatocytes,[63] and with *in vitro* studies demonstrating that the activation GS involves a *p21-ras*–independent pathway.[65-66] Thus, our results provide insights into the biochemical mechanisms of insulin-signalling in the liver.

Effect of* p21-ras *Upon Enzymes That Are Known To Be Altered in Preneoplastic Foci. Histochemical assays have been used to identify "enzyme-altered hepatic foci" in carcinogen-treated rodents. These are considered to be preneoplastic lesions, although they can spontaneously regress, and have never been definitively proven to develop into cancers.[67-69] G6Pase, OCT, and canalicular ATPase are frequently inactivated in enzyme-altered hepatic foci, while 5′NT, G6PD, and α-GPD are frequently activated. All of these changes were induced by *Ras-gal* in animals that did not receive a carcinogen, suggesting that the activation of *p21-ras,* or of any upstream or downstream mediators, might induce these changes. Other enzymatic changes that can be observed in

FIG. 4. Effect of activated *p21-ras* upon liver functions in hepatocytes from the livers of *Ras-gal*–transduced DEN-untreated rats and in HCC from *Ras-gal*–transduced DEN-treated rats. (A-R) Represent enzymatic assays performed on liver biopsy specimens that were obtained at several months after transduction with *Ras-gal* from the livers of DEN-untreated rats. (A and B) OCT. OCT activity results in a brown color. (A) Arrows indicate three G6Pase-negative foci that are deficient in OCT (B) at 5 months after transduction. (×100) (C and D) NADPH-reductase. NADPH-reductase activity results in a blue color. (C) Arrows indicate three G6Pase-negative foci that are deficient in NADPH-reductase (D) at 5 months after transduction. (×100) (E and F) β-HBD. β-HBD activity results in a blue color. (E) Arrows indicate a G6Pase-negative focus that is deficient in β-HBD (F) at 10 months after transduction. (×200) (G and H) Canalicular ATPase. Canalicular ATPase results in a brown color at the canalicular membrane. (G) Arrows indicate two G6Pase-negative foci that are deficient in canalicular ATPase (H) at 10 months after transduction. (×100) (I and J). SD activity results in a blue color. (I) Arrows indicate three G6Pase-negative foci that have normal levels of SD (panel J) at 10 months after transduction. (×100) (K and L) Glyceraldehyde 3-phosphate dehydrogenase (G3PD). G3PD activity results in a blue color. (K) Arrows indicate two G6Pase-negative foci that have normal levels of G3PD (L) at 10 months after transduction. (×40) (M and N) ICDH. ICDH activity results in a blue color. (M) The arrow identifies a G6Pase-negative focus that has markedly increased ICDH activity (N) at 10 months after transduction. (×100) (O and P) G6PD. G6PD activity results in a blue color. (O) The arrows identify two G6Pase-negative foci that had strong activation of G6PD (P) at 10 months after transduction. (×40) (Q and R) 5′ Nucleotidase (5′ NT). 5′NT activity results in a brown color at the canalicular membrane. (Q) Arrows indicate three G6Pase-negative foci that have increased 5′NT activity (R) at 10 months after transduction. (×100) (S-X) Histochemical assays of liver sections obtained from the HCC of the livers from *Ras-gal*–transduced DEN-treated rats. Both *cancer 1546* and *cancer 1566* were primarily HCC, as assessed by HE staining. (S-V) Histochemical stains of *cancer 1546.* γGT (S), ALP (T), and lipase (U) all result in a brown color when enzymatic activity is present. The arrows indicate region B, which has strong activation of γGT, ALP and lipase. In contrast, region B has lost expression of GK (V), as indicated by arrows that identify a region that is not blue. (W and X) Histochemical stains of *cancer 1566.* (W) ICDH Enzymatic activity results in a blue color. The arrows indicate region B, which abruptly lost expression of ICDH. Region B has strong activation of ALP, as shown in (X).

the enzyme-altered hepatic foci, such as activation of γGT, were not induced by *Ras-gal*.

***Miscellaneous Enzymes Whose Expression Is Altered by* p21-ras *in Hepatocytes* In Vivo.** The histochemical assay known as NADPH-diaphorase, NADPH-tetrazolium reductase, or NADPH-cytochrome P450 reductase probably represents a cytochrome P450 reductase,[70] while NADH-tetrazolium reductase is probably related to NADH dehydrogenase.[39] Both of these activities were strongly inhibited in the *Ras-gal*–transduced foci of DEN-untreated rats. The inhibition of NADPH or NADH reductase might enhance the carcinogenic potential of a drug by preventing its inactivation through reduction. Alternatively, the inactivation of these enzymes might prevent the activation of a drug to a toxic metabolite, thus inhibiting its carcinogenic potential. Thus, the inhibition of the NADPH and NADH reductases might alter the frequency of additional mutations that are necessary for the development of cancers in the multistep model of carcinogenesis.

Biochemical Explanation for the Enzymatic Changes That Are Induced in* Ras-gal–*Transduced Foci. The biochemical explanation for the changes that were induced by the activated *p21-ras* is unclear. The activation of *p21-ras* results in the activation of members of the *Fos* and *Jun* family, that dimerize to form *AP-1* and transcriptionally activate several genes that are involved in replication.[26] In contrast, *AP-1* can inhibit the albumin enhancer in *Ras*-transformed *SV40*-immortalized hepatocytes.[71] Thus an increase in *AP-1* activity in *Ras-gal*–transduced foci could activate some promoters or enhancers and downregulate others. Alternatively, *p21-ras* might downregulate the liver-specific transcription factors, leading to a generalized loss of hepatic functions.

TABLE 1. Results of Histochemical Assays

Enzyme	Effect of Insulin or Feeding in Literature*	Effect in Enzyme-Altered Hepatic Foci in the Literature†	Result in *Ras-gal*–Transduced DEN-Untreated Cells‡	Effect in Liver Cancer in the Literature§	Cancer 1566‖		Cancer 1546	
					A	B	A	B
Glycogen Metabolism								
Phosphorylase	↓[72,73]	↓,↑[74-76]	No Δ	↓[75,76]	–	NP	–	–
Glycogen Syn	↑[72,73]	↓, ↑[75,76]	No Δ	↑, ↓[75,76]	+	NP	+	–
Gluconeogenesis								
Glucose-6-Pase	↓[72]	↓[75]	↓↓	↓↓↓[77]	–	–	–	–
Glycolysis								
Glucokinase	↑[72,21]	↓[75]	↑	↓↓[77]	+	NP	+	–
Hexokinase	No Δ	↑[75]	No Δ	↑[79]	+	NP	+	–
P-fructokinase	↑[78]		↑	↑[77]	+	+	+	+/–
Aldolase	↑[78]		No Δ	↓[77]	+	+/–	+	+
Glyceraldehyde-3-P-De	↑[78,73,74]	↑[74,75]	No Δ	↑[75,76]	+	NP	+	–
Pyruvate kinase	↑[78,73,74]	↓[75]	No Δ	↑↑[76,77]	+	NP	–	–
Citric Acid Cycle								
Isocitrate De	↓[80]		↑↑↑		++	–	++	–
Succinate De	No Δ[81]	↑, ↓[76,82]	No Δ	↑, ↓[76]	+	+	+	+
Malic De	↑[80]	↑[75,76]	↑	↑, ↓[76]	+	–	+	–
Lipid Synthesis								
Glucose 6-P De	↑[72,21]	↑[75]	↑↑	↑↑↑[77]	+	–	+	–
6-P-Gluconate De	↑[72,73,80]	↑[75]	↑	↓[77]	+	–	–	–
α-Glycerol-P-De	↑[73]	↑[75]	↑↑	↓[77]	+	–	+	–
Lipases								
Carbon 12			No Δ		–	NP	–	+
Carbon 16-18			No Δ		+/–	+/–	+/–	+
Ketogenesis								
β-hydroxybut. De	↓[72]		↓↓	↓↓[83]	+/–	–	–	–
Membrane-Associated								
5′ Nucleotidase		↑[74]	↑↑	↓[85]	–	+/–	+/–	+
Canalicular ATPase		↑, ↓[74,82]	↓↓↓	No Δ[82]	–	–	–	–
Nonlocalized ATPase		↑, ↓[82]	No Δ	↑, ↓[76]	+	NP	+	+
Adenylate Cyclase		↓[75,76]	No Δ	↑, ↓[75,76]	+	NP	++	–
Alkaline Pase		↑, ↓[74,82]	↑*	↑, ↓[76,82]	+	NP	+	++
Amino acid metabolism								
Glutamate De	↓[85]; ↑[76]		No Δ	↑[76]	+	+	+	+
γ-GT		↑[22]	No Activity	↑[76]	–	NP	–	+
Other Enzymes								
NADH Reductase			↓		+/–	–	+/–	–
NADPH Reductase		↑, ↓[69]	↓↓		+/–	–	+/–	–
Lactate De		No Δ[82]	↑	No Δ, ↑[84,85]	+	–	+	–
OCT		↓[77]	↓↓	↓↓[23,25]	–	NP	–	–
UDPG-De			↑		+	NP	+	–

NOTE. * (↑) indicates that insulin and/or feeding increased enzyme activity in animals according to the literature; and (↓) indicates inhibition. † (↑) indicates that the enzyme can be activated in enzyme-altered hepatic foci of carcinogen-fed animals according to the literature; and (↓) indicates inactivation. ‡ The effect of *p21-ras* upon liver functions in hepatocytes *in vivo* in this study in DEN-untreated rats. (↑) indicates activation in 100% of at least 10 foci while (↓) indicates inhibition. A greater amount of arrows indicates a stronger response; and (↑*) indicates that ALP was only activated in 50% of the foci. § The published activity of enzymes in HCC. (↑) indicates that the enzyme is more active in cancerous liver than in normal liver; and (↓) indicates inhibition. ‖ The result of enzyme assays for HCC from the livers of *Ras-gal*–transduced DEN-treated rats. For both cancers, the region designated "A" was similar to the *Ras-gal*–transduced hepatocytes. The region designated "B" differed in a number of properties. For *Tumor 1566,* region B was absent from several slides as the block was progressively sectioned, as indicated by NP. (++) indicates that the enzymatic activity was higher than in normal liver; (+) indicates activity similar to normal liver; (+/–) indicates that activity was present but less than normal liver; and (–) indicates that enzyme activity was completely absent.

Abbreviations: De, dehydrogenase; NP, not present; OCT, ornithine carbamoyltransferase; Pase, phosphatase; Syn, synthase; transp, transpeptidase.

Δ; change.

CCAAT-enhancer binding protein is critical for the transcription of several gluconeogenic enzymes[65] and can inhibit the replication of and promote the differentiation of adipocytes.[8] Although in their study Hu and Isom[70] failed to observe the downregulation of CCAAT-enhancer binding protein or of other transcription factors in *Ras*-tranduced/*SV40*-immortalized hepatocytes in culture, the effects of *p21-ras* in culture might not reflect what occurs *in vivo*. *Ras-gal*–transduced foci are currently being tested to determine if *AP-1* is activated and/or if liver-specific transcription factor genes are inactivated.

HCC Have Regions That Lose Hepatic Cell Functions and Activate Bile Ductule/Oval Cell Functions. Two of the previously-described HCCs from the livers of *Ras-gal*–transduced DEN-treated rats[26] were also analyzed. Region A of both HCCs resembled the *Ras-gal*–transduced foci from the livers of the DEN-untreated rats, while region B was dramatically different. Region B of *cancer 1546* expressed genes that are characteristic of the bile ductule cells, such as ALP and γGT,[72] and lost the expression of hepatic genes. Similarly, region B of *cancer 1566* expressed ALP and lost the expression of many hepatic genes, although it did not express γGT. This abrupt loss of liver-specific genes, and the activation of bile ductule/oval-cell genes suggests that a single genetic event that superimposed upon the other mutations that resulted in a malignant phenotype might be responsible. This enzymatic data is consistent with the fact that one cholangiocarcinoma and one mixed HCC/cholangiocarcinoma developed from the *Ras-gal*–transduced DEN-treated rat livers, as determined by special stains of formalin-fixed cancers.[26] This putative mutation might represent the loss of a gene that promotes differentiation along the hepatic lineage, or the activation of a gene that promotes differentiation along the bile ductule pathway. Attempts to identify this putative differentiation-inducing gene are in progress.

REFERENCES

1. Anderson MW, Reynolds SH, You M, Maronpot RM. Role of proto-oncogene activation in carcinogenesis. Environ Health Perspect 1992;98:13-24.
2. Lea MA. Regulation of gene expression in hepatomas. Int J Biochem 1993; 25:457-469.
3. Unsal H, Yakicier C, Marcais C, Kew M, Volkmann M, Zentgraf H, Isselbacher KJ, et al. Genetic heterogeneity of hepatocellular carcinoma. Proc Natl Acad Sci U S A 1994;91:822-826.
4. Wands IR, Blum HE. Primary hepatocellular carcinoma. N Engl J Med 1991;325:729-731.
5. Harris H. The role of differentiation in the suppression of malignancy. J Cell Sci 1990;97:5-10.
6. Auersperg N, Roskelley C. Retroviral oncogenes: interrelationships between neoplastic transformation and cell differentiation. Crit Rev Oncog 1991;2:125-136.
7. Uriel J. Cancer, retrodifferentiation, and the myth of Faust. Cancer Res 1976;36:4269-4275.
8. Umek RM, Friedman AD, McKnight SL. CCAAT/enhancer binding protein: a component of a differentiation switch. Science 1991;251:288-292.
9. Love JM, Gudas LJ. Vitamin A, differentiation and cancer. Curr Opin Cell Biol 1994;6:825-831.
10. Coleman WB, Wennerberg AE, Smith GJ, Grisham JW. Regulation of the differentiation of diploid and some aneuploid rat liver epithelial (stemlike) cells by the hepatic microenvironment. Am J Pathol 1993;142:1373-1382.
11. Board M, Colquhoun A, Newsholme EA. High Km glucose-phosphorylating activities in a range of tumor cell lines and inhibition of rates of tumor growth by the specific enzyme inhibitor mannoheptulose. Cancer Res 1995; 55:3278-3285.
12. Hill CS, Treisman R. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 1995;80:199-211.
13. Medema RH, Bos JL. The role of p21-ras in receptor tyrosine kinase signaling. Crit Rev Oncog 1993;4:615-661.
14. Lee G-H, Li H, Ohtake K, Nomura K, Hino O, Furata Y, Aizawa S, et al. Detection of activated c-H-ras oncogene in hepatocellular carcinomas developing in transgenic mice harboring albumin promoter-regulated SV40 gene. Carcinogenesis 1990;11:1145-1148.
15. Beer D, Schwarz M, Sawada N, Pitot HC. Expression of H-ras and c-myc proto-oncogenes in isolated GGT-positive rat hepatocytes and in hepatocellular carcinomas induced by DEN. Cancer Res 1986;46:2435-2441.
16. Buchmann A, Bauer-Hoffman R, Mahr J, Drinkwater NR, Luz A, Schwartz M. Mutational activation of the c-Ha-ras gene in liver tumors of different rodent strains: correlation with susceptibility to hepatocarcinogenesis. Proc Natl Acad Sci U S A 1991;88:911-915.
17. Chandar N, Lombardi B, Schulz W, Locker J. Analysis of ras genes and linked viral sequences in rat hepatocarcinogenesis. Am J Pathol 1987;129: 232-241.
18. Tokusashi Y, Fukada I, Ogawa K. Absence of p53 mutations and various frequencies of Ki-ras exon 1 mutations in rat hepatic tumors induced by different carcinogens. Mol Carcinog 1994;10:45-51.
19. Yaswen P, Goyette M, Shank PR, Fausto N. Expression of c-Ki-ras, c-Ha-ras, and c-myc in specific cell types during hepatocarcinogenesis. Mol Cell Biol 1985;5:780-786.
20. Chen B, Liu L, Castonguay A, Maronpot RR, Anderson MW, You M. Dose-dependent ras mutation spectra in N-nitrosodiethylamine induced mouse liver tumors and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induced mouse lung tumors. Carcinogenesis 1993;14:1603-1608.
21. Tada M, Omata M, Ohto M. Analysis of ras gene mutations in human hepatic malignant tumors by polymerase chain reaction and direct sequencing. Cancer Res 1990;50:1121-1124.
22. Tsuda H, Hirohashi S, Shimosato Y, Ino Y, Yoshida T, Terasa M. Low incidence of point mutations of c-Ki-ras and N-ras oncogenes in human hepatocellular carcinoma. Jpn J Cancer Res 1989;80:196-199.
23. Ogato N, Kamimura T, Asakura H. Point mutation, allelic loss and increased methylation of c-Ha-ras gene in human hepatocellular carcinoma. HEPATOLOGY 1991;13:31-37.
24. Gu J-R, Hu L-F, Cheng Y-C, Wan D-F. Oncogenes in human primary hepatic cancer. J Cell Physiol 1986;4(suppl):13-20.
25. Richards CA, Short SA, Thorgeirrson SS, Huber BE. Characterization of a transforming N-ras gene in the human hepatoma cell line Hep G2: additional evidence for the importance of c-myc and ras cooperation in hepatocarcinogenesis. Cancer 1990;50:1521-1527.
26. Lin Y-Z, Brunt E, Bowling W, Hafenrichter DG, Kennedy S, Flye MW, Ponder KP. Ras-transduced diethylnitrosamine-treated hepatocytes develop into cancers of mixed phenotype *in vivo*. Cancer Res 1995;55:5242-5250.
27. Rettinger S, Kennedy S, Wu X, Flye MW, Ponder KP. Liver-directed gene therapy: quantitative evaluation of promoter elements using *in vivo* retroviral transduction. Proc Natl Acad Sci U S A. 1994;91:1460-1464.
28. Ghattas IR, Sanes JR, Majors JE. The encephalomyocarditis virus internal ribosome entry site allows efficient co-expression of two genes from a recombinant provirus in cultured cells and in embryos. Mol Cell Biol 1991; 11:5848-5857.
29. Yan C, Costa R, Darnell J, Chen J, Van Dyke T. Positive and negative elements control the limited hepatocyte and choroid plexus expression of transthyretin in transgenic mice. EMBO J 1990;9:869-878.
30. McCoy MS, Bargmann CI, Weinberg RA. Human colon carcinoma Ki-ras-2 oncogene and its corresponding proto-oncogene. Mol Cell Biol 1984;4: 1577-1582.
31. Soriano P, Montgomery C, Geske R, Bradley A. Targeted disruption of the c-src protooncogene leads to osteopetrosis in mice. Cell 1991;64:693-702.
32. Markowitz D, Goff S, Bank A. Construction and use of a safe and efficient amphotropic packaging cell line. Virology 1988;167:400-406.
33. Jainchill JL, Aaronson SA, Todaro GJ. Murine sarcoma and leukemia viruses: assay using clonal lines of contact inhibited mouse cells. J Virol 1969;4:549-553.
34. Rettinger S, Ponder KP, Saylors R, Kennedy S, Hafenrichter D, Flye MW. *In vivo* transduction with retrovirus during in-flow occlusion. J Surg Res 1993;54:418-425.
35. Sheehan DC, Hrapchak BB. Theory and practice of histotechnology. St. Louis: Mosby, 1980:144;164-166;205.
36. Sasse D. Histology and histochemistry. In: Thurman RG, Kauffman FC, Jungermann K, eds. Regulation of hepatic metabolism. New York: Plenum, 1986:78-83.
37. Thomas E, Pearse AGE. The fine localization of dehydrogenases in the nervous system. Histochemie 1961;2:266-282.
38. Jacobsen NO, Jørgensen F. Further enzyme histochemical observations on the segmentation of the proximal tubules in the kidney of the male rat. Histochemie 1973;34:11-32.
39. Lojda Z, Gossrau R, Scheibler TH. Enzyme histochemistry: a laboratory manual. Berlin: Spronger-Verlag, 1979:223-269.
40. Chayen J, Bitensky L, Butcher RG, eds. Practical histochemistry. London: Wiley, 1973:111-196.
41. Rieder H, Teutsch HF, Sasse D. NADP-dependent dehydrogenases in rat liver parenchyma. Histochem Cell Biol 1978;56:283-298.
42. Stevens A. Enzyme histochemistry: diagnostic applications. In: Bancroft JD, Stevens A, eds. Theory and practice of histological techniques. Edinburgh: Churchill Livingstone, 1990:1:401-411.
43. Gomori G. Microscopic histochemistry: principles and practice. Chicago: University of Chicago Press, 1952.
44. Chiquoine AD. Distribution of G6Pase in the liver of the mouse. J Histochem Cytochem 1953;1:429-435.
45. Wachstein M, Meisel E. Histochemistry of hepatic phosphatases at a physiologic pH. Am J Clin Pathol 1957;13-23.
46. Mizutani A. Cytochemical demonstration of ornithine carbamoyltransferase activity in liver mitochondria of rat and mouse. J Histochem Cytochem 1968;16:172-180.
47. Mayer D, Hacker HJ, Bannasch P. Re-evaluation of the specificity of ade-

nylyl (β,γ-methylene)diphosphonate as a substrate for adenylate cyclase. Histochem J 1991;23:100-106.
48. Rutenburg AM, Kim H, Fischbein JW, Hanker JS, Wasserkrug HL, Seligman AM. Histochemical and ultrastructural demonstration of γ-glutamyl transpeptidase activity. J Histochem Cytochem 1969;17:517-526.
49. Lawrence GM, Trayer IP, Walker DG. Histochemical and immunohistochemical localization of hexokinase isoenzymes in normal rat liver. Histochem J 1984;16:1099-1111.
50. Henderson B. Quantitative cytochemical measurement of glyceraldehyde 3-phosphate dehydrogenase activity. Histochem Cell Biol 1976;48:191-204.
51. Klimek F, Moore MA, Schneider E, Bannasch P. Histochemical and microbiochemical demonstration of reduced pyruvate kinase activity in thioacetamide-induced neoplastic nodules of rat liver. Histochem Cell Biol 1988;90:37-42.
52. Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. Biotechniques 1989;7:980-987.
53. MacGregor GR, Nolan GP, Fiering S, Roederer M, Herzenberg LA. Use of *E. coli* lac Z (β-galactosidase) as a reporter gene. In: Murray EJ, Walker JM, eds. Methods in molecular biology. Vol 7. Totowa: Humana Press Inc, 1993:317-327.
54. Ponder KP, Gupta S, Leland F, Darlington G, Finegold M, DeMayo J, Ledley F, et al. Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation. Proc Natl Acad Sci U S A 1991;88:1217-1221.
55. Ohe K, Morris HP, Weinhouse S. β-Hydroxybutyrate dehydrogenase activity in liver and liver tumors. Cancer Res 1967;27:1360-1371.
56. Lee G-H, Merlino G, Fausto N. Development of liver tumors in transforming growth factor α transgenic mice. Cancer Res 1992;52:5162-5170.
57. Sandgren EP, Quaife CJ, Pinkert CA, Palmiter RD, Brinster RL. Oncogene-induced liver neoplasia in transgenic mice. Oncogene 1989;4:715-724.
58. Okuda K, Kojiro M, Okuda H. Neoplasms of the liver. In: Schiff L, Schiff ER, eds. Diseases of the liver. 7th ed. Philadelphia: Lippincott, 1993:1236-1265.
59. Ruch RJ, Madhukar BV, Trosko JE, Klaunig JE. Reversal of *ras*-induced inhibition of gap-junctional intercellular communication, transformation, and tumorigeneses by lovastatin. Mol Carcinog 1993;7:50-59.
60. Kohl NE, Wilson FR, Mosser SD, Giuliani E, DeSolms SJ, Conner MW, Anthony NJ. Protein farnesyltransferase inhibitors block the growth of *ras*-dependent tumors in nude mice. Proc Natl Acad Sci U S A 1994;91:9141-9145.
61. Tamanoi F. Inhibitors of Ras farnesyltransferases. Trends Biochem Sci 1993;18:349-353.
62. Alpers DH, Sabesin SM, White HM. Fatty liver: biochemical and clinical aspects. In: Schiff L, Schiff ER, eds. Diseases of the liver. 7th ed. Philadelphia: Lippincott, 1993;2:825-855.
63. Lee J, Pilch PF. The insulin receptor; structure, function, and signaling. Am J Physiol 1994;266:C319-C334.
64. Vaulont S, Kahn A. Transcriptional control of metabolic regulation genes by carbohydrates. FASEB J 1994;8:28-35.
65. Lin TA, Lawrence JC. Activation of ribosomal protein S6 kinase does not increase glycogen synthetase or glucose transport in rat adipocytes. J Biol Chem 1994;269:21255-21261.
66. Sakaue H, Hara K, Noguchi T, Matozaki T, Kotani K, Ogawa W, Yonezawa K, et al. Ras-independent and wortmannin sensitive activation of glycogen synthetase by insulin in Chinese hamster ovary cells. J Biol Chem 1995;270:11304-11309.
67. Pitot HC. Altered hepatic foci: their role in murine hepatocarcinogenesis. Annu Rev Toxicol 1990;30:465-500.
68. Bannasch P, Mayer D, Hacker H-J. Hepatocellular glycogenesis and hepatocarcinogenesis. Biochem Biophys Acta 1980;605:217-245.
69. Williams GM. The pathogenesis of rat liver cancer caused by chemical carcinogens. Biochem Biophys Acta 1980;605:167-189.
70. Buchmann A, Kuhlmann W, Schwartz M, Kunz W, Wolf CR, Moll E, Friedberg T, et al. Regulation and expression of four cytochrome P-450 isoenzymes, NADPH-cytochrome P-450 reductase, the glutathione transferases B and C and microsomal epoxide hydrolase in preneoplastic and neoplastic lesions in rat liver. Carcinogenesis 1985;6:513-521.
71. Hu J, Isom HC. Suppression of albumin enhancer activity by H-ras and AP1 in hepatocyte cell lines. Mol Cell Biol 1994;14:1531-1543.
72. Sirica AE, Mathis GA, Sano N, Elmore LW. Isolation, culture, and transplantation of intrahepatic biliary epithelial cells and oval cells. Pathobiology 1990;58:44-64.
73. Seifter S, Englard S. Energy metabolism. In: Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter D, Shafritz DA, eds. The liver: biology and pathobiology. New York: Raven, 1994;323-364.
74. Denton RM, Tavare JM. Mechanisms whereby insulin may regulate intracellular events. In: Ashcroft FM, Ashcroft SJH, eds. Insulin: molecular biology to pathology. New York: IRL Press, 1992:235-256.
75. Pitot HC. Altered hepatic foci: their role in murine hepatocarcinogenesis. Annu Rev Pharmacol Toxicol 1990;30:465-500.
76. Mayer D, Klimek F, Hacker HJ, Bannasch P. Carbohydrate metabolism in hepatic pre-neoplasia. In: Bannasch P, Keppler D, Weber G, eds. Liver cell carcinoma. Boston: Kluwer Academic Publishers, 1988:329-346.
77. Toshkov I, Chisari FV, Bannasch P. Hepatic preneoplasia in hepatitis B virus transgenic mice. HEPATOLOGY 1994;20:1162-1172.
78. Weber G. Enzymology of cancer cells. N Engl J Med 1977;296:541-551.
79. O'Brian RM, Granner DK. Regulation of gene expression by insulin. Biochem J 1991;278:609-619.
80. Klimek F, Bannasch P. Isoenzyme shift from glucokinase to hexokinase is not an early but a late event in hepatocarcinogenesis. Carcinogenesis 1993;14:1857-1861.
81. Rieder H. NADP-dependent dehydrogenases in rat liver parenchyma. Histochem Cell Biol 1981;72:579-615.
82. Nepokroeff CM, Lakshmanan MR, Ness GC, Muesing RA, Kleinsek DA, Porter JW. Coordinate control of rat liver lipogenic enzymes by insulin. Arch Biochem Biophys 1974;162:340-344.
83. Kitagawa T. Histochemical analysis of hyperplastic lesions and hepatomas of the liver of rats red 2-fluorenylacetamide. Jpn J Cancer Res 1971;62:207-216.
84. Weber G. Biochemical strategy of hepatocellular carcinoma. In: Bannasch P, Keppler D, Weber G, eds. Liver cell carcinoma. Boston: Kluwer Academic Publishers, 1988:361-374.
85. Sapag-Hagar M, Lagunas R, Sols A. Apparent unbalance between the activities of 6-phosphogluconate and glucose-6-phosphate dehydrogenases in rat liver. Biochem Biophys Res Commun 1973;50:179-185.