Jpn. J. Cancer Res. 88, 1155–1164, December 1997

Prohibitin Expression Is Decreased in the Regenerating Liver but Not in Chemically Induced Hepatic Tumors in Rats

Satoshi Tanno, Ikue Fukuda, Yoshinori Saito and Katsuhiro Ogawa[1]

Department of Pathology, Asahikawa Medical College, 4-5-3-11 Nishikagura, Asahikawa 078

Expression of prohibitin, a growth-regulatory protein, was immunohistochemically investigated in normal rat tissues, regenerating livers, and chemically induced preneoplastic and neoplastic hepatic lesions. Specific cell types including hepatocytes, striated and smooth muscle cells, cardiac cells, squamous epithelial cells, sebaceous gland cells, hair root outer sheath cells, salivary gland duct epithelial cells, chondrocytes, immature spermatocytes and oocytes were found to be positive. In regenerating livers, prohibitin protein disappeared as early as 3 h after two-thirds hepatectomy and returned to near the original level by 24 h, while its mRNA level did not markedly vary. The timing of the disappearance was coincident with the expression of c-*myc*, suggesting a relation to quiescent hepatocytes entering the cell cycle. However, no pronounced decrease was evident in the most hyperplastic hepatic nodules and hepatocellular carcinomas investigated. Examination of 9 rat hepatocellular carcinoma cell lines, 6 hyperplastic hepatic nodules and 5 hepatocellular carcinomas revealed a single case of a base substitution in prohibitin cDNA, identified as a synonymous sense change. The observed abundant expression of prohibitin in quiescent hepatocytes and its rapid loss under conditions of regeneration indicate a growth-regulatory function, but our results do not suggest any critical role in rat hepatocarcinogenesis.

Key words: Prohibitin — Liver regeneration — Hepatocarcinogenesis

The prohibitin (*PHB*) gene, cloned from fractionated mRNA of normal rat liver, has the ability to inhibit proliferation of human diploid fibroblasts after microinjection.[1–4] The *PHB* gene is present in various species including mammals, *Drosophila* and yeast.[5, 6] Microinjection of its mRNA leads to growth inhibition in normal and tumor cells, while suppression of *PHB* expression by PHB antisense oligonucleotide, in contrast, stimulates proliferation.[7–9] PHB amino acid sequences deduced from cDNA sequences have been found to be almost identical in human, mice and rats, with high homology to those of *Drosophila* and yeast.[5, 10] In addition, a gene encoding another protein with high homology to PHB has been identified in mice, suggesting the existence of a *PHB* gene family.[11] Although its exact function is currently unknown, PHB has been localized to the mitochondrial outer membrane[12] and, in the case of mouse B lymphocytes, to the IgM antigen receptor site,[11] suggesting some role in mitochondrial functions and signal transduction involving IgM.

Structural changes of the *PHB* gene have been detected in some human breast cancers, indicating that loss of its growth-suppressive function may play a role in neoplasia.[13, 14] However, although Sato *et al.*,[14] Cliby *et al.*[15] and Asamoto and Cohen[16] noted that *PHB* mutations are absent in human liver, ovarian and lung cancers and rat bladder tumors, PHB has not otherwise been extensively studied. On the other hand, the presence of *PHB* mRNA has been demonstrated in various rat tissues as well as human cell lines,[5–7] but it remains largely unknown what cell types normally express PHB and whether the levels may change during cell proliferation *in vivo*. In the present study, we therefore immunohistochemically investigated its expression in normal rat tissues and regenerating livers, as well as hyperplastic hepatic nodules (HPNs) and hepatocellular carcinomas (HCCs) induced by chemical carcinogens in rats. In addition, we examined structural changes in PHB cDNA in rat HCC cell lines and chemically induced hepatic lesions. The results demonstrated that PHB is expressed in particular cell types of normal rat tissues, including hepatocytes, and that it disappears from the latter during the early phase of liver regeneration. However, we found no evidence of appreciable loss of PHB in HPNs and HCCs, and *PHB* mutations were not detected.

MATERIALS AND METHODS

Tissues and cells Various normal tissues were isolated from 7 male and 6 female F344 rats (5 rats, 1–5 days and 8 rats, 6–24 weeks old), following perfusion-fixation with periodate-lysine-paraformaldehyde[17] through the left heart ventricle or portal vein. Regenerating livers were removed from 48 6-week-old male F344 rats at various periods after two-thirds hepatectomy. As controls, 12 animals underwent sham-operation, in which the livers were manipulated under ether anesthesia as in the case of partial hepatectomy, but without resection. HPNs and

[1] To whom correspondence should be addressed.

HCCs were induced by treatment of 20 male F344 rats according to the Solt and Farber protocol[18] [a single dose of diethylnitrosamine (100 mg/kg body weight), followed two weeks later by dietary 2-acetylaminofluorene for 2 weeks, and a two-thirds hepatectomy at the 3rd week]. Nine rat HCC cell lines were obtained from the Japanese Resource Bank for Cancer Research.

PHB antibody A PHB oligopeptide, Ile-Phe-Asp-Cys-Arg-Ser-Arg-Pro-Arg-Asn-Val-Pro-Val (residues 66–78), was synthesized with reference to the reported sequence of rat PHB cDNA,[7] and polyclonal antibody was raised by immunizing New Zealand white rabbits with the peptide. The antibody was purified by affinity chromatography using the PHB oligopeptide.

Immunohistochemistry The tissues were routinely processed for paraffin-embedding, and 4 μm sections were incubated with the PHB antibody, followed by visualization of antibody binding using a Histofine kit (Nichirei, Tokyo). Preimmune rabbit serum was used as a negative control. The tissues were also immunostained for proliferating cell nuclear antigen (PCNA) and/or glutathione-*S*-transferase placental form (GST-P), the latter being a marker for preneoplastic or neoplastic rat hepatocytes.[19] The antibody against PCNA was purchased from Dako Patt SA (Glostrup, Denmark), and the anti-GST-P antibody was a kind gift from Prof. Kiyomi Sato of Hirosaki University.

Western blotting analysis Normal rat liver tissues were solubilized in buffer containing 50 m*M* HEPES (pH 7.4), 250 m*M* NaCl, 1 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40, and samples were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, then electroblotted onto nitrocellulose membranes. The membranes were incubated with the PHB antibody, and binding was detected using a chemiluminescence reagent (Renaissance, DuPont NEN, Boston, MA).

Northern blot analysis A PHB DNA probe including the entire coding region of PHB cDNA was produced by reverse transcriptase-polymerase chain reaction (PCR) from normal rat liver mRNA and cloned into a TA cloning vector (Invitrogen, San Diego, CA). mRNA was isolated from hepatic tissues of 32 normal, partially hepatectomized or sham-operated rats using a Fast track mRNA isolation kit (Invitrogen), blotted onto nitrocellulose membranes and hybridized with the ^{32}P-dCTP labeled PHB probe. The same membranes were then used for hybridization with a c-myc probe (Oncogene Science, Uniondale, NY) as well as with a β-actin probe to permit normalization of RNA loading.

Structural analysis of PHB cDNA mRNA was isolated from HPN and HCC tissues and HCC cell lines using a Fast Track mRNA isolation kit. First strand cDNA was synthesized by reverse transcriptase, and the entire coding region of PHB cDNA was amplified by PCR using a set of primers (Fig. 1, Table I). Then, six segments overlapping with each other were amplified by nested PCR using the primers, one of which contained a GC clamp,[20] and the products were analyzed by denaturing gradient gel electrophoresis (DGGE) as described elsewhere.[21] Briefly, the PCR amplified DNA from the test samples was mixed with the corresponding wild-type fragments and underwent a cycle of denaturation-annealing to produce heteroduplex DNA consisting of wild type and mutated strands, followed by DGGE. The range of denaturant concentrations at which the wild

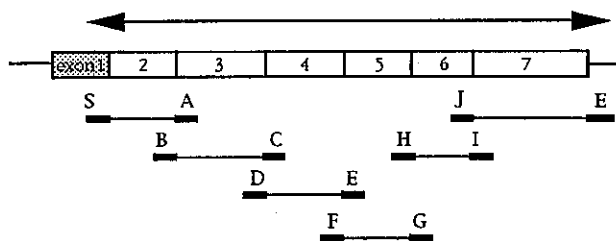

Fig. 1. PCR-amplification of PHB cDNA. After amplification of the entire translated region of PHB cDNA (arrowed bar), small overlapping fragments were generated by nested PCR. One of each set of primers used for the nested PCR contained a GC-clamp.

Table I. PCR Primers Used for Amplification of PHB cDNA

Sense	Antisense
S; 5′-GC clamp[a]-TTCCTCAGTACCGACGGTTT-3′	A; 5′-TGTGATGTTGACATTCTGCAA-3′
B; 5′-GC clamp-CAGTAGTGGCCGTCGTTTCTG-3′	C; 5′-CAATTCTCCAGCATCGAATC-3′
D; 5′-GC clamp-TGTCTCTAGGAGTTCAGCCA-3′	E; 5′-TCCCGAAGGTCAGATGTGT-3′
F; 5′-GC clamp-TAGGACCTACTGCACAGGGA-3′	G; 5′-TCACCCTCAGCAGAGATGAT-3′
H; 5′-GC clamp-CGACTCGTCGTCTTCTTCCG-3′	I; 5′-TGATAAGCAATGTCCTCAGC-3′
J; 5′-GC clamp-CTCGACGCTTTCGACCTTCG-3′	E; 5′-CAGAAGGAAGGCGTCATTCA-3′

a) GC clamp: 5′-GCGGGCGGGGCGGGGGCACGGGGGGCGCGG-3′.

type PCR fragments showed a conformation change was first determined for each kind of fragment by perpendicular DGGE. Then, the samples were simultaneously analyzed by parallel DGGE. When a pattern suggesting the presence of a mutation was observed, the PCR fragments were inserted into a TA vector, and their nucleotide sequences were determined.

RESULTS

Expression of PHB in normal rat tissues A single 30 kD protein was detected by immunoblotting analysis using the PHB antibody in lysates of normal rat liver (Fig. 2); its molecular weight coincides with that of PHB predicted from its cDNA structure.[3] Expression of PHB was immunohistochemically examined in normal tissues of 3 male and 2 female newborn rats, and 4 male and 4 female adult rats of various ages. The data are summarized in Table II. In the liver, PHB was prominently expressed in normal hepatocytes, whereas bile duct cells and sinusoidal cells were negative (Fig. 4a). PHB was also detected in various other cells including muscle cells (striated, smooth and cardiac), squamous epithelium (epidermis and esophagus), sebaceous gland epithelium, outer sheath epithelium of hair roots, excretory duct epithelium of the salivary glands, chondrocytes, oocytes and immature spermatocytes (Fig. 3, a–h). The staining pattern did not differ between male and female rats except for the gonadal tissues. Expression patterns of PHB were also similar between adults and newborns in most tissues except for the lung, where alveolar epithelium was only very weakly stained in adults, but was clearly positive in newborns (Fig. 3, i and j). PHB was

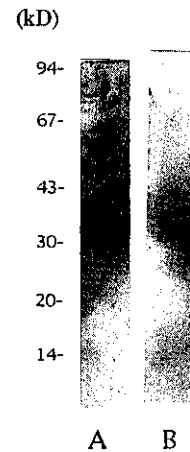

Fig. 2. Western blot analysis with the anti-PHB antibody showing a single 30 kD protein in a lysate of normal rat liver (A). Control using the preimmune rabbit serum (B).

Table II. Expression of PHB in Normal Rat Tissues

Organs or tissues	Intensity[a)]	Cell types
Central nervous system	−	
Skin	+	outer root sheath cells, sebaceous cells, keratinocytes
Skeletal muscle	++	striated muscle cells
Salivary glands	+	excretory dust epithelium
Esophagus	+	keratinizing epithelium, smooth muscle cells
Stomach & Intestine	+	smooth muscle cells
Liver	+++	hepatocytes
Pancreas	−	
Trachea & Bronchi	+	chondrocytes
Lung	+	alveolar epithelium[b)]
Kidney	−	
Testis	+	immature spermatocytes
Ovary	++	oocytes
Oviduct & Uterus	+	smooth muscle cells
Blood vessels	+	smooth muscle cells
Heart	++	cardiac muscle cells
Lymph nodes	−	
Spleen	−	
Thymus	−	
Thyroid gland	−	
Hypophysis	−	
Adrenals	−	

a) Staining intensity; negative (−), weakly positive (+), intermediate (++) and strongly positive (+++).
b) Positive in newborn, but negative or only weakly positive in adults.

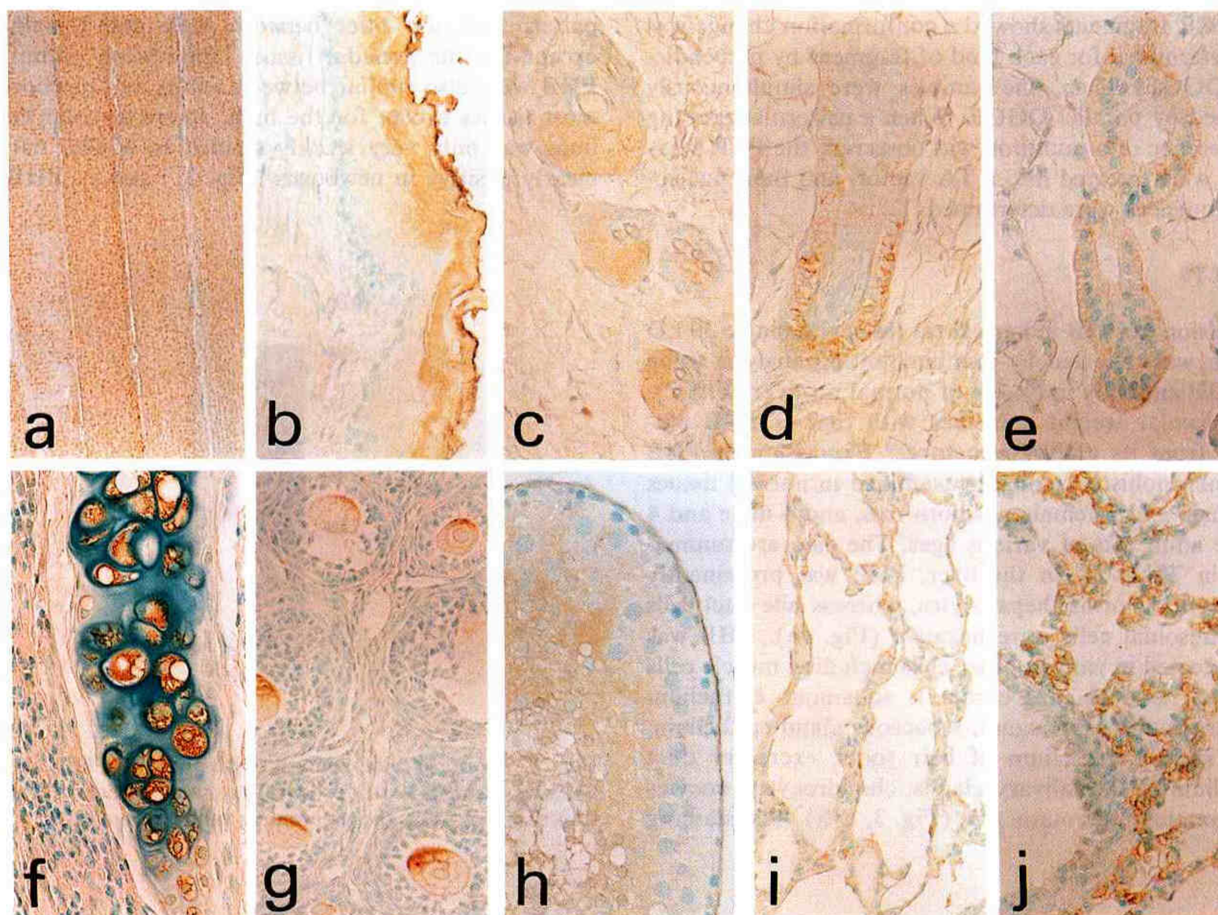

Fig. 3. Immunostaining for PHB in various normal rat tissues. Cardiac cells (a), keratinocytes in the esophagus (b), sebaceous gland cells (c), outer sheath cells of hair roots (d), duct epithelium of the submandibular salivary gland (e), chondrocytes in the tracheal cartilage (f), oocytes (g), spermatogenic cells (h), alveolar epithelium of an adult lung (i) and alveolar epithelium of a newborn lung (j). (a–g, i and j, ×100; h, ×200)

always granularly stained in the cytoplasm, and no nuclear or cell membrane localization was observed. No positive staining was detected when preimmune serum was used instead of the PHB antibody.

***PHB* expression in regenerating livers** Expression of *PHB* in regenerating livers was immunohistochemically investigated using at least 3 animals at each time point after partial hepatectomy. PHB staining became almost completely negative in most hepatocytes during the 3–12 h period, returning to near normal levels at 24 h (Fig. 4, b and c). PHB first disappeared from the periportal areas of the liver lobules, whereas it initially reappeared in the centrilobular areas. Although the staining intensity fluctuated thereafter, it became normal after 10 days. Northern blot analysis did not reveal any marked drop in PHB mRNA in regenerating livers (Fig. 5). Two bands of 1.2 and 1.9 kb were observed in all the test samples as previously described,[7] with the 1.9 kb band being much weaker. Although c-*myc* mRNA was very low in the normal liver, it was elevated during the period between 3–6 h after partial hepatectomy, roughly coinciding with the decrease in PHB protein. The results of northern blot analysis were almost the same in three repeated experiments using samples isolated from different animals. Expression of PCNA was elevated after 24 h (data not shown), indicating that a large fraction of the cells entered S phase during this period, as described in the literature.[22] In sham-operated rats, *PHB* staining was decreased during a period of 3–6 h after the operation but to a lesser degree than in the group given partial hepatectomy (Fig. 4d). The PHB mRNA level was not significantly changed. Interestingly, c-*myc* mRNA was also detected in sham-operated livers at 3 h.

PHB in HPN and HCC tissues At 3 weeks after the start

Fig. 4. PHB immunohistochemistry in the normal rat liver (a), and regenerating liver at 3 h (b) and 6 h (c) after two-thirds hepatectomy and 6 h after sham-operation (d). PHB is abundantly expressed in the hepatocytes in the normal liver, but sinusoidal and bile duct cells are negative (a). During liver regeneration, PHB is markedly decreased 3 and 6 h after hepatectomy (b, c). PHB is also decreased in the liver at 6 h after sham-operation, but to a lesser extent than in the case of two-thirds hepatectomy (d). (×100)

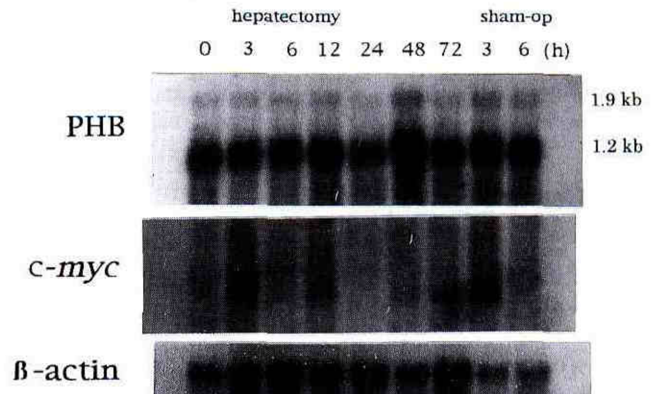

Fig. 5. Northern blot analysis of PHB, c-*myc* and β-actin in normal, regenerating and sham-operated livers. Expression of PHB mRNA is unchanged after partial hepatectomy and sham-operation. In contrast, c-*myc* is negative in the normal liver, but becomes positive in regenerating livers 3–6 h after hepatectomy. It is also apparent in the sham-operated liver at 3 h after the operation.

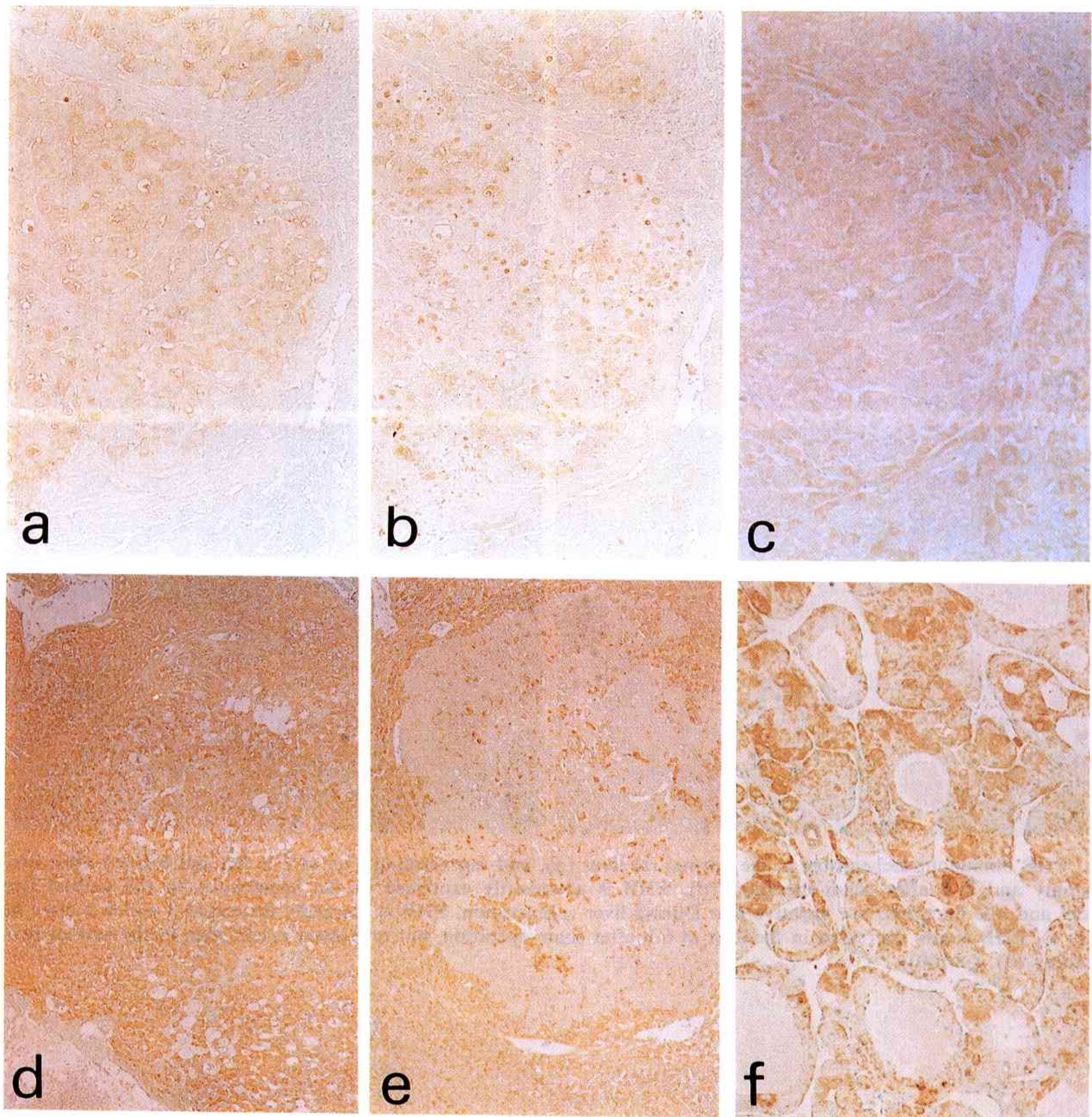

Fig. 6. Immunohistochemical staining of GST-P, PCNA and PHB in neoplastic and preneoplastic hepatocytes. An early HPN (4 weeks after the start of the carcinogenic treatment) is positive for GST-P (a) and PCNA (b), while the surrounding normal hepatocytes are negative. The PHB staining in the HPN is comparable to that in the surrounding liver (c). Late HPNs (d, e) and an HCC (f) (24–36 weeks after the start of treatment). PHB is present at levels almost comparable to those in the surrounding liver in (d) and (f), whereas a marked decrease is evident in (e). (a–c, ×100; d and e, ×40; f, ×130)

of the carcinogenic treatment, small foci of carcinogen-altered hepatocytes,[22] which were positive for GST-P but almost negative for PCNA, showed PHB staining almost to the same degree as the surrounding normal hepatocytes (data not shown). Grossly visible GST-P-positive HPNs which showed many PCNA-positive cells 4–6 weeks after the start of carcinogenic treatment (Fig. 6, a and b) also demonstrated the same or only slightly weaker PHB staining as compared with the surrounding normal hepatocytes (Fig. 6c). After 10–24 weeks, HPNs were larger but contained fewer PCNA-positive cells. Most did not differ from the surrounding liver with

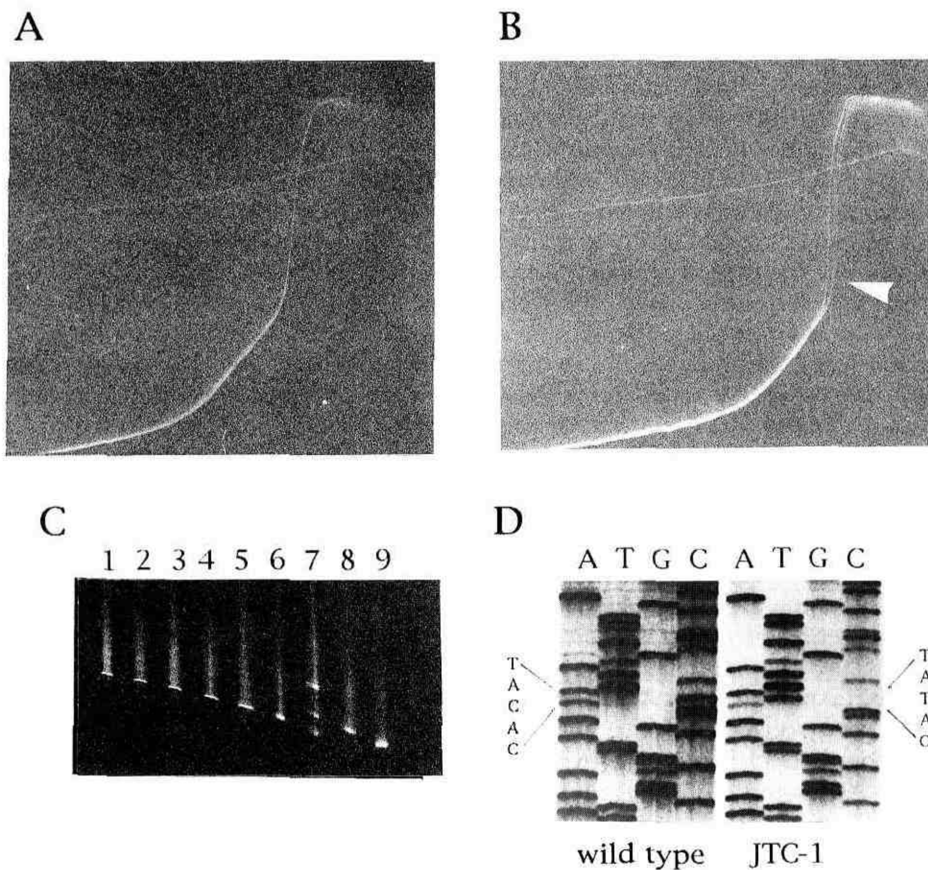

Fig. 7. DGGE analysis of PHB cDNA. Perpendicular DGGE of the PCR fragments including PHB exon 4 for normal liver (A) and that for JTC-1 cell line plus normal liver (B). An abnormal line (arrow) is seen in the DGGE of JTC-1 cells in association with the wild-type line. Parallel DGGE analysis of exon 4 fragments of various HCC cell lines (C). Morris D plus wild type (lane 1), Morris D alone (lane 2), dRLh84 plus wild type (lane 3), dRLh84 alone (lane 4), FAA-HTC1 plus wild type (lane 5), FAA-HTC1 alone (lane 6), JTC-1 plus wild type (lane 7), JTC-1 alone (lane 8) and the normal rat liver (lane 9). An abnormal pattern is apparent for JTC-1 cells plus wild type (lane 7). DNA sequencing reveals a C-to-T change at codon 107 in the JTC-1 cells (D).

regard to PHB staining (Fig. 6d), although some PHB-negative cells were observed in a few cases. A very few HPNs were almost entirely negative for PHB (Fig. 6e), but these were not different from other lesions in their morphological features or in their proliferative activity as estimated by PCNA staining (data not shown). Most HCCs were positive to the same degree as the surrounding liver tissues, but a few showed decreased PHB staining (Fig. 6f).

Structure of PHB cDNA from HPN and HCC tissues, and HCC cell lines GC-clamped PCR products were generated from 6 segments of PHB cDNA, which included translated exons,[7] for 9 HCC cell lines, 5 HPNs and 6 HCCs. The DGGE method revealed only one case with an abnormal pattern. This involved exon 4 in the JTC-1 cell line, suggesting the presence of a base change (Fig. 7, A–C). Although nucleotide sequencing revealed that there was a base substitution (C to T) at codon 107 (Fig. 7D), this was a synonymous-sense mutation not associated with any amino acid substitution.

DISCUSSION

The *PHB* gene was identified as encoding a growth-inhibitory factor by injection of mRNAs from normal rat liver into human diploid fibroblasts.[1-4] A growth-suppressive function for PHB was demonstrated in human diploid fibroblasts and tumor cells.[7-9] Recently, Jupe *et al.*[8, 9] reported that the growth inhibition is specific to group B among immortal complementation groups A, B, C and D as defined by Pereira-Smith and Smith.[23] PHB cDNA has two poly adenylation signals which result in

two mRNAs, of 1.2 and 1.9 kb,[7] both of which are ubiquitously expressed in normal rat tissues as well as human cell lines.[7] The present immunohistochemical study, however, demonstrated that PHB protein is not uniformly expressed, but rather is limited to specific cell types within normal rat tissues. Since PHB was identified in cells with and without proliferation potential, its expression is not strictly related to a loss of ability to divide. The fact that PHB staining was always granular in the cytoplasm, is consistent with the recent report of Ikonen *et al.*[12] that PHB is localized in mitochondria.

The distribution pattern of PHB protein in this study is not in complete accordance with that reported for PHB mRNA.[7] For example, although the brain, spleen and kidney were shown to contain abundant PHB mRNA, to the same extent as the liver,[7] no PHB-positive cells were immunohistochemically detectable in these tissues. The discrepancy between immunostaining of PHB protein and mRNA expression may be explained if a small amount of PHB protein, under the threshold level for immunohistochemical detection, is present in many cells. Alternatively, it is possible that multiple antigenically different forms may be expressed in a tissue-specific manner. Liu *et al.*[24] have recently detected multiple forms of PHB with various isoelectric points but with the same molecular weight. Although PHB is thought not to be modified by glycosylation or fatty acid complementation,[7] there is a possibility that PHB is post-translationally modified by phosphorylation, methylation, sulfation and/or mono-ADP-ribosylation and that our antibody might not recognize all the forms.

PHB staining was detected in immature spermatocytes in testis, whereas it was negative in spermatogonia and mature spermatocytes, as described by Choongkittaworn *et al.*[25] Our current study further revealed its abundant expression in oocytes, indicating that PHB may have a function in gonadal cells in both males and females. The *Drosophila* Cc gene, a homologue of the *PHB* gene, is expressed in late stage embryos, but to a lesser degree in early embryos, late third instar larvae and adults.[10] Since flies with nonfunctional Cc gene die during the larva-to-pupa metamorphosis,[10] the Cc gene must play an important role in development and differentiation of *Drosophila*. In this respect, it is noteworthy that alveolar epithelium of the lung was found to be positive for PHB in newborns, while being negative or only very weakly positive in adult rats. Clearly, the function of PHB during mammalian development *in utero* warrants attention.

In the present study, PHB staining rapidly disappeared in hepatocytes after a two-thirds hepatectomy, this first occurring in the periportal areas, consistent with the established fact that regeneration starts in zone 1 of the acinus.[26,27] The coincident timing with elevation of c-*myc* mRNA is consistent with a link to quiescent hepatocytes entering the cell cycle. This is in line with the recent report by Roskams *et al.*[28] that anti-proliferative activity of microinjected PHB mRNA is prominent in the G0/G1 phase of the cell cycle, while it decreases as the cells approach the S phase. Although levels of PHB mRNA were not significantly changed at 3, 6, 12, 24 and 48 h after partial hepatectomy in the present study, Lumpkin *et al.*[29] reported temporary 3-fold elevation at 0.5–1 h and 8–12 h. Although the discrepancy might be explained by the timing of sampling of the materials and/or variance in northern blotting quantitation, the expression of PHB mRNA does not seem to decrease as much as PHB protein. Thus, the disappearance of PHB immunostaining may be due, at least partly, to a rapid breakdown of the protein or a masking of its antigenic epitope. Considering the reported growth-suppressive function of PHB, its loss may be an important event during the early phase of liver regeneration, together with expression of various oncogenes and growth factor genes.[30,31] On the other hand, Lumpkin *et al.*[29] reported that gavage with ethanol one hour prior to partial hepatectomy resulted in marked elevation of PHB mRNA during 0.5–2 h after surgery, as well as retardation of liver regeneration. It would clearly be of interest to determine whether the immunostaining of PHB is retained under such conditions.

It is noteworthy that PHB protein was decreased and c-*myc* mRNA was increased in sham-operated rats in the present study. An increase in the portal vein pressure was reported to increase c-*myc* mRNA to a level comparable to that after a two-thirds hepatectomy in isolated-perfused liver.[32] Such changes in c-*myc* and PHB may be caused by circulatory or other changes associated with the sham-operation.

The finding of normal levels of *PHB* expression in most HPNs and HCCs induced by Solt and Farber's protocol,[18] with rare exceptions, is not suggestive of a major role in hepatocarcinogenesis. Moreover, the PHB cDNA structure was not altered in most HCC cell lines, and no mutations were found in HPN and HCC tissues, suggesting that PHB expressed in those tissues is functionally normal. Our data, and the reports of abundant *PHB* expression in rat urinary bladder carcinomas and their cell lines with no detectable mutations,[16] suggest that PHB should not be considered a tumor suppressor gene.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Kiyomi Sato for providing the antibody to GST-P. This work was funded by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

(Received August 6, 1997/Accepted October 6, 1997)

REFERENCES

1) Lumpkin, C. K., Jr., McClung, J. K. and Smith, J. R. Entry into S phase is inhibited in human diploid fibroblasts by rat liver poly(A)+RNA. *Exp. Cell Res.*, **160**, 544–549 (1985).
2) Lumpkin, C. K., Jr., McClung, J. K., Pereira-Smith, O. M. and Smith, J. R. Existence of high abundance antiproliferative mRNA's in senescent human diploid fibroblasts. *Science*, **232**, 393–395 (1986).
3) McClung, J. K., Danner, D. B., Stewart, D. A., Smith, J. R., Schneider, E. L., Lumpkin, C. K., Jr., Dell'Orco, R. and Nuell, M. J. Isolation of a cDNA that hybrid selects antiproliferative mRNA from rat liver. *Biochem. Biophys. Res. Commun.*, **164**, 1316–1322 (1989).
4) Nuell, M. J., McClung, J. K., Smith, J. R. and Danner, D. B. Approach to the isolation of antiproliferative genes. *Exp. Gerontol.*, **24**, 469–476 (1989).
5) McClung, J. K., Jupe, E. R., Liu, X.-T. and Dell'Orco, R. T. Prohibitin: potential role in senescence, development, and tumor suppression. *Exp. Gerontol.*, **30**, 99–124 (1995).
6) Dell'Orco, R. T., McClung, J. K., Jupe, E. R. and Liu, X.-T. Prohibitin and the senescent phenotype. *Exp. Gerontol.*, **31**, 245–252 (1996).
7) Nuell, M. J., Stewart, D. A., Walker, L., Friedman, V., Wood, C. M., Owens, G. A., Smith, J. R., Schneider, E. L., Dell'Orco, R., Lumpkin, C. K., Jr., Danner, D. B. and McClung, J. K. Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. *Mol. Cell. Biol.*, **11**, 1372–1381 (1991).
8) Jupe, E. R., Liu, X.-T., Kiehlbauch, J. L., McClung, J. K. and Dell'Orco, R. T. Prohibitin antiproliferative activity and lack of heterozygosity in immortalized cell lines. *Exp. Cell Res.*, **218**, 577–580 (1995).
9) Jupe, E. R., Liu, X.-T., Kiehlbauch, J. L., McClung, J. K. and Dell'Orco, R. T. The 3′ untranslated region of prohibitin and cellular immortalization. *Exp. Cell Res.*, **224**, 128–135 (1996).
10) Eveleth, D. D., Jr. and Marsh, J. L. Sequence and expression of the *Cc* gene, a member of the dopa decarboxylase gene cluster of *Drosophila*: possible translational regulation. *Nucleic Acids Res.*, **15**, 6169–6183 (1986).
11) Terashima, M., Kim, K. M., Adachi, T., Nielsen, P. J., Reth, M., Kohler, G. and Lamers, M. C. The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. *EMBO J.*, **13**, 3782–3792 (1994).
12) Ikonen, E., Fiedler, K., Parton, R. G. and Simons, K. Prohibitin, an antiproliferative protein, is localized to mitochondria. *FEBS Lett.*, **358**, 273–277 (1995).
13) Sato, T., Saito, H., Swensen, J., Olifant, A., Wood, C., Danner, D., Sakamoto, T., Takita, K., Kasumi, F., Miki, Y., Skolnick, M. and Nakamura, Y. The human prohibitin gene located on chromosome 17q21 is mutated in sporadic breast cancer. *Cancer Res.*, **52**, 1643–1646 (1992).
14) Sato, T., Sakamoto, T., Takita, K., Saito, H., Okui, K. and Nakamura, Y. The human prohibitin (*PHB*) gene family and its somatic mutations in human tumors. *Genomics*, **17**, 762–764 (1993).
15) Cliby, W., Sarkar, G., Ritland, S. R., Hartmann, L., Podratz, K. C. and Jenkins, R. B. Absence of prohibitin gene mutation in human epithelial ovarian tumors. *Gynecol. Oncol.*, **50**, 34–37 (1993).
16) Asamoto, M. and Cohen, S. M. Prohibitin gene is overexpressed but not mutated in rat bladder carcinomas and cell lines. *Cancer Lett.*, **83**, 201–207 (1994).
17) McLean, I. W. and Nakane, P. K. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.*, **22**, 1077–1083 (1974).
18) Solt, D. and Farber, E. New principle for the analysis of chemical carcinogenesis. *Nature*, **263**, 701–703 (1976).
19) Satoh, K., Hatayama, I., Tateoka, N., Tamai, K., Shimizu, T., Tatematsu, M., Ito, N. and Sato, K. Transient induction of single GST-P positive hepatocytes by DEN. *Carcinogenesis*, **10**, 2107–2111 (1989).
20) Sheffield, V. C., Cox, D. R., Lerman, L. S. and Myer, M. Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl. Acad. Sci. USA*, **86**, 232–236 (1989).
21) Fukuda, I. and Ogawa, K. Detection of *p53* gene mutations in rat hepatocellular carcinoma cell lines by denaturing gradient gel electrophoresis. *Mol. Carcinog.*, **7**, 257–262 (1993).
22) Ogawa, K., Solt, D. and Farber, E. Phenotypic diversity as an early property of putative preneoplastic hepatocyte populations in liver carcinogenesis. *Cancer Res.*, **40**, 725–733 (1980).
23) Pereira-Smith, O. M. and Smith, J. R. Genetic analysis of indefinite division in human cells: identification of four complementation groups. *Proc. Natl. Acad. Sci. USA*, **85**, 6042–6046 (1988).
24) Liu, X.-T., Stewart, C. A., King, R. L., Danner, D. A., Dell'Orco, R. T. and McClung, J. K. Prohibitin expression during cellular senescence of human diploid fibroblasts. *Biochem. Biophys. Res. Commun.*, **201**, 409–414 (1994).
25) Choongkittaworn, N. M., Kim, K. H., Danner, D. B. and Griswold, M. D. Expression of prohibitin in rat seminiferous epithelium. *Biol. Reprod.*, **49**, 300–310 (1993).
26) Grisham, J. W. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-^{3}H. *Cancer Res.*, **22**, 842–849 (1962).
27) Rabes, H. M. Kinetics of hepatocellular proliferation as a function of the microvascular structure and functional state of the liver. *In* "Hepatotrophic Factors," ed. R.

Porter and J. Whelan, pp. 31–53 (1977). Excerpta Medica, Amsterdam.
28) Roskams, A. J., Friedman, V., Wood, C. M., Walker, L., Owens, G. A., Stewart, D. A., Altus, M. S., Danner, D. B., Liu, X.-T. and McClung, J. K. Cell cycle activity and expression of prohibitin mRNA. *J. Cell. Physiol.,* **157,** 289–295 (1993).
29) Lumpkin, C. K., Jr., Moore, T. L., Tarpley, M. D., Taylor, J. M., Badger, T. M. and McClung, J. K. Acute ethanol and selected growth suppressor transcripts in regenerating liver. *Alcohol,* **12,** 357–362 (1995).
30) Faust, N. and Mead, J. E. Biology of disease. Regulation of liver growth; protooncogenes and transforming growth factors. *Lab. Invest.,* **60,** 4–13 (1989).
31) Michalopoulos, G. Liver regeneration. *Science,* **276,** 60–66 (1997).
32) Isomura, H., Sawada, N., Nakajima, Y., Sakamoto, H., Ikeda, T., Kojima, T., Enomoto, K. and Mori, M. Increase in portal flow induced c-myc expression in isolated perfused rat liver. *J. Cell. Physiol.,* **154,** 329–332 (1993).