Overexpression of a *p*-Glycoprotein in Hepatocellular Carcinomas From Woodchuck Hepatitis Virus–Infected Woodchucks (*Marmota monax*)

SANDRA E. DUNN,[1] CHRISTINE S. HUGHES,[2] GERALD A. LEBLANC,[1] AND JOHN M. CULLEN[3]

The leading cause of human hepatocellular carcinomas (HCCs) is hepatitis B virus (HBV) infection. Woodchucks infected with a closely related hepadnavirus, woodchuck hepatitis virus (WHV), serve as a model for HBV because woodchucks chronically infected with WHV also develop hepatocellular carcinomas. Increased expression of *p*-glycoprotein (pgp) in human HCCs is a common obstacle in successful cancer chemotherapy. Pgps are encoded by a family of multidrug-resistance (MDR) genes. Livers from uninfected and WHV-infected woodchucks were examined to determine if pgp was expressed in HCCs and if there was a difference in expression between HCCs and nonneoplastic liver. A 170-kd protein was identified by Western blot in HCCs, whereas, constitutive pgp was not detected in normal liver taken from the same animals in 3 of 3 cases. Immunolocalization of the pgp with a panel of monoclonal antibodies revealed intensification of staining in 7 of 20 foci and 12 of 22 HCCs from six animals. Using primers for the human MDR1 gene, a single product was detected by reverse-transcribed polymerase chain reaction (RT-PCR) from HCCs. We have shown an increase in pgp in HCCs compared with normal liver from WHV-infected woodchucks. This is the first example of the induction of a pgp in a naturally hepadnavirus infected rodent system. It suggests the woodchuck can be a useful model for the study of the acquisition of resistance to chemotherapeutic agents in virally induced HCCs. (HEPATOLOGY 1996;23:662-668.)

Drug resistance is a frequent clinical problem in the successful treatment of cancer.[1-5] Pleotropic drug resistance has been closely associated with the overexpression of multidrug-resistance (MDR) genes[5] and has been observed in hepatocellular carcinomas (HCCs), making the prognosis for patients with HCC very poor.[6] HCC is one of the most common cancers in the world[7]; therefore, it is important to understand the mechanisms of drug resistance in hopes of developing better therapeutic modalities in the treatment of HCC.

The overexpression of MDR genes that code for a *p*-glycoprotein (pgp) has been shown to correlate with levels of drug resistance in both cell culture[8] and human liver biopsy samples.[9,10] In normal hepatocytes, pgp functions as a secretory pump that provides a protective mechanism against cytotoxic plant alkaloids, dietary xenobiotics,[11] and chemical carcinogens such as benzo(a)pyrene[12] and possibly 2-amino-acetylfluorene.[13] There is a second class of pgp found in human liver that is encoded by MDR2, the function of which has not been determined. The mouse homolog, mdr2, has been shown to be involved in lipid trafficking.[14] In mice and rats, there are three MDR genes, mdr1, mdr2, and mdr3, but only mdr1 and mdr3 appear to participate in drug resistance.[15,16]

There are estimates of approximately 450,000 new cases per year of human cancers overexpressing MDR1 gene,[9] some of which are associated with a viral agent such as hepatitis B virus (HBV).[17] Chronic HBV infection is a major risk factor for HCC[18] with a global distribution of approximately 300 million HBV carriers.[18] The woodchuck (*Marmota monax*) is a valuable rodent model for studying hepatocarcinogenesis because woodchucks are the natural host for an HBV-like virus, the woodchuck hepatitis virus (WHV). WHV-infected woodchucks serve as a useful model for studying viral carcinogenesis because: (1) woodchucks, like humans, develop HCC during chronic infection with a hepadnavirus, (2) the WHV is hepatotropic, hence tumors are liver specific, and (3) HBV and WHV viral DNA sequences are 70% homologous.[19] In this study, we have examined nonneoplastic, preneoplastic, and neoplastic samples from WHV-infected woodchuck liver to determine if: (1) woodchuck liver expressed pgp, (2) pgp expression was increased during neoplastic transformation, and (3) a MDR1-like gene that codes for pgp could be detected.

MATERIALS AND METHODS

Animals. Woodchucks were trapped from southeastern Pennsylvania and obtained from Northeastern Wildlife (South Plymouth, NY). Animals were housed individually, or

Abbreviations: MDR, multidrug resistance; HCC, hepatocellular carcinomas; pgp, *p*-glycoprotein; HBV, hepatitis B virus; WHV, woodchuck hepatitis virus; Mab, monoclonal antibodies; PBS, phosphate-buffered saline; RT-PCR, reverse-transcribed polymerase chain reaction; cDNA, complementary DNA; PCR, polymerase chain reaction; mRNA, messenger RNA.

From the [1]Department of Toxicology; [2]Department of Anatomy, Physiological Sciences, and Radiology; and [3]Department of Microbiology, Pathology, and Parasitology, North Carolina State University, College of Veterinary Medicine, Raleigh, NC.

Received August 30, 1994; accepted November 3, 1995.

Research funding was supported in part by a grant from the State of North Carolina.

Address reprint requests to: John M. Cullen, North Carolina State University, College of Veterinary Medicine, 4700 Hillsborough St., Raleigh, NC 27606.

0270-9139/96/2304-0002$3.00/0

in group runs at the North Carolina State University College of Veterinary Medicine laboratory animal facility. The age of the animals could not be determined because they were wild caught as adults. Animals were fed a primate leaf-eater diet (Agway, Marion, KS) ad libitum, and maintained on a photoperiod that corresponded to natural light cycles. All animal manipulations were approved by the North Carolina State University, Raleigh, NC, Animal Care and Use Committee.

WHV infection was naturally acquired, and the status of infection was monitored by routine blood sampling. Serology for markers of WHV infection were performed by enzyme-linked immunosorbent assay through a generous collaboration with Dr. Paul Cote, Georgetown University, Washington, D.C., according to published methods.[20-22] Viral DNA was detected by slot blot analysis using a probe consisting of the complete WHV genome.[22]

Woodchucks were chemically restrained with Innovar-Vet (Pitman-Moore, Mundelein, IL) and killed by a lethal intravenous injection of pentobarbital (Henry Schein, Melville, NY). Livers were either frozen in liquid nitrogen and stored at −70°C or fixed in neutral buffered formalin and paraffin embedded for further investigation.

Western Blot. Two unrelated mouse monoclonal antibodies (Mab) were used to identify the putative woodchuck pgp. These Mab react with different, mutually exclusive cytoplasmic epitopes of pgp based upon reciprocal blocking experiments.[23] JSB-1 (Biogenics, San Ramon, CA) is a murine Mab, subclass immunoglobulin G1 which binds a cytoplasmic epitope of the Chinese hamster ovary cell line pgp.[24] C219 (Signet, Dedham, MA) is an immunoglobulin G2 murine Mab for chinese hamster ovary pgp that also binds a highly conserved cytoplasmic epitope.[24] Canine kidney was used as a positive control because it has been shown to cross-react with C219.[25] Fresh canine kidney is routinely stained positive with JSB-1 and C219 (personal communication, Page R, College of Veterinary Medicine, North Carolina State University, February 1995).

Crude membrane preparations were isolated according to Bell et al.[26] Protein concentrations were determined using the BioRad Protein Assay (Biorad, Hercules, CA). Separation of 50 μg of proteins was performed on a 5.6% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Crude membranes were screened for pgp using a Signet elite Avidin-biotin pgp detection kit (Signet). Blots were blocked with 3% bovine serum albumin, followed by incubation with JSB-1 or C219 at a 1:1000 dilution. A goat anti-mouse alkaline phosphatase conjugated secondary antibody (Sigma Chemical Co, St. Louis, MO) was incubated at 1:5,000 for 1 hour.

For low level detection of pgp, chemiluminescent detection was performed using a Amersham ECL method (Amersham Life Sciences, Amersham, England). This application was employed to detect small amounts of pgp in the analysis of tumor and normal WHV-infected liver. Proteins (10 μg) were separated by a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The nitrocellulose was incubated overnight at 4°C in 10% nonfat dried milk dissolved in phosphate-buffered saline (PBS). This step was done as a blocking step to minimize nonspecific binding. JSB-1 was diluted 1:5,000 and incubated for 1 hour. The blot was incubated with a rabbit anti-mouse horseradish peroxidase conjugated antibody (Sigma Chemical Company) at a dilution of 1:10,000 for 1 hour. The amount of protein used in the Western blot assay was determined to be in the linear range of detection by protein titration (data not shown) which was quantitated by laser densitometry using a Silverscanner II (LeCie Ltd., Beaverton, OR).

Immunohistochemistry. Samples from nine WHV-infected nonneoplastic liver biopsies, two uninfected liver samples, or six HCC from WHV-infected liver were serially sectioned. Duplicate sections were either stained with hematoxylin-eosin or subjected to immunohistochemical staining. A panel of Mab (JSB-1, C219, and C494) was used to investigate the expression of pgp. C494 (Signet), a Mab to the chinese hamster ovary pgp, was included in the panel because it binds specifically to human MDR1 gene products.[27] Sections of formalin-fixed, paraffin-embedded liver were blocked with 5% normal goat serum in PBS for 30 minutes. Mabs were incubated with the liver sections (1:100 in PBS) overnight at room temperature, and the sections were coverslipped to prevent dehydration. Before treatment with the secondary antibody, the sections were rinsed three times in PBS. Goat anti-mouse immunoglobulin alkaline phosphatase conjugated antibody (Sigma) was applied to the sections at a 1:50 dilution in PBS and incubated for 30 minutes. Slides were then rinsed three times in PBS for 30 minutes. BCIP/NTB (5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium), a substrate for alkaline phosphatase, was used as a chromagen. Sections were counter-stained with nuclear fast red, dehydrated, cleared, and mounted. Nonimmune serum and nontumorous tissues were used as negative controls.

Semiquantitative Reverse-Transcribed Polymerase Chain Reaction. Total RNA was isolated from liver using a Promega RNagents kit (Promega, Madison, WI) according to Chomczynski.[28] RNA was subjected to guanine isothiocyanide treatment followed by a phenol/chloroform extraction. RNA was precipitated in the presence of isoamyl alcohol, centrifuged at 10,000*g* and the resulting pellet was washed in ethanol. RNA was resuspended in a Tris-ethylenediaminetetraacetic acid pH 7.4 buffer and stored at −135°C. Samples were subjected to reverse-transcribed polymerase chain reaction (RT-PCR) according to a modified protocol from Noonan et al.[29] All reagents for RT-PCR were purchased from Promega unless otherwise stated. RNA was serially diluted to 2.0, 1.0, 0.5, 0.25, 0.125 μg/RT reaction. Before reverse transcription, RNA was treated with 25 pmol oligo dT at 72°C for 10 minutes then placed immediately on ice. Reverse transcription of total RNA was performed in a 20-μL master mix (1× RT buffer [Gibco BRL, Gaithersburg, MD], 12.5 mmol/L DTT, 2.5 mmol/L each dNTP, 14 U RNAsin, 100 U Superscript reverse transcriptase [Gibco BRL]). The samples were reverse transcribed 42°C for 15 minutes, 99°C for 5 minutes, and 5°C for 5 minutes. The resulting complementary DNA (cDNA) was diluted 4× and 10 μL was taken for PCR. A 40-μL PCR master mix (1× PCR buffer, 200 mmol/L each dNTP, 1.8 mmol/L $MgCl_2$, 1.25 units Taq DNA polymerase, 250 ng of each MDR primer) was prepared for each sample. The cDNA was amplified by PCR 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 2 minutes using the following primers synthesized by DNAgency (Ashton, PA). MDR sense-CCCATCATTGCAATAGCAGG and MDR antisense-GTTCAAACTTCTGCTCCTGA were derived from the human MDR1 gene.[30] Assuming that the PCR was more than 90% efficient, 25, 12.5, 6.25, 3.125, 1.563 ng cDNA was generated per PCR reaction. PCR products (10 μL) were electrophoresed on a 4% agarose gel, yielding a single 167-bp fragment. Ethidium bromide stained gels were photographed and the band intensities were quantitated on a Silverscann II laser densitometer. RNA from a HepG2 cell line was used as a positive control. Reagent controls were run as negative controls (data not shown). There was a decrease in band intensity with decreasing amounts of template cDNA; therefore, the reactions were below saturation. Samples were normalized to the amount of RNA loaded using band intensity of the 28 S RNA.

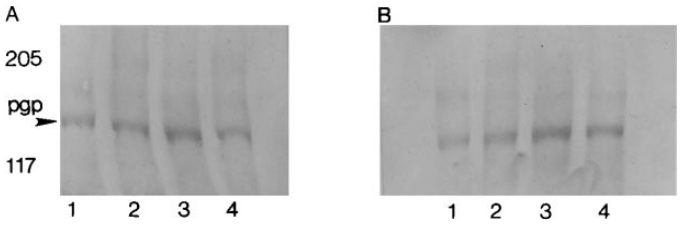

FIG. 1. A colorimetric Western blot analysis using JSB-1 (blot A) and C219 (blot B) to probe crude membrane isolates, which were separated on a 5.6% polyacrylamide gel. Canine kidney was used as a positive control in lane 1. Lanes 2 through 4 were HCC from three woodchucks, HC019, HC018, and HC032. To each lane, 50 μg of total protein was loaded. The molecular weight of the woodchuck pgp was estimated to be ~170 kd based on migration of known standards.

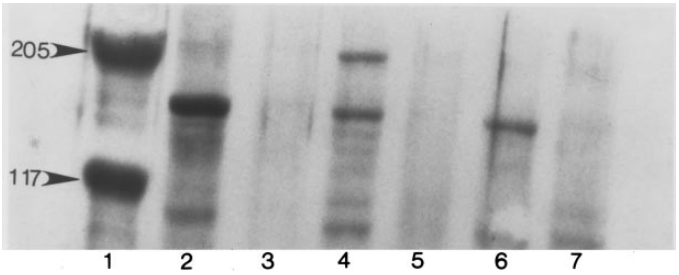

FIG. 2. Chemiluminescent Western blot analysis of crude membrane isolates from HCC and nonneoplastic liver separated on a 7.5% polyacrylamide gel and probed with JSB-1. Prestained molecular-weight standards of 205 and 117 kd were run in lane 1. Lanes 2 and 3, 4 and 5, and 6 and 7 represent HCC and nonneoplastic, WHV-infected liver from HC019, HC037, and HC017, respectively. A total protein concentration of 10 μg was loaded to each lane, which was determined to be in the linear range by titrating 2.5 to 20 μg of total protein and analyzing by Western blot analysis (data not shown).

Southern Blot. RT-PCR was performed as above with the following modifications, PCR products were electrophoresed on a 4% agarose gel, yielding a single 167-bp fragment. The cDNA products were transferred to a nylon membrane and baked at 80°C for 1 hour. The blot was prehybridized for 1 hour at 65°C (5× Denharts reagent, 5× SSPE, 0.2% SDS, and 500 μg/mL ssDNA). A 24mer cDNA probe was generated (DNAgency) based upon human sequence internal to the primers used for PCR.[30] The probe was ^{32}P-labeled with a random primer kit (Gibco BRL). Hybridization was initiated by the addition of the probe directly into the prehybridization solution. The blot was hybridized overnight at 65°C. Unhybridized radioactivity was washed from the blot using 5 mmol/L sodium phosphate, 1 mmol/L ethylenediaminetetraacetic acid, and 0.2% sodium dodecyl sulfate at room temperature three times at 30-minute intervals. The membrane was placed on film for 24 hours at −70°C.

RESULTS

To determine if pgp was expressed in woodchuck liver tumors, HCC from WHV-infected animals were screened by Western blot analysis using JSB-1 and C219 (Fig. 1). Both antibodies recognized a 170-kd protein in three HCC taken from three woodchucks HC019, HC018, and HC032, respectively. The putative woodchuck pgp comigrated with pgp from canine kidney. Both antibodies bound to a protein of the predicted molecular weight, and the degree of binding was similar for both Mab, as observed in the liver of other rodents.[24]

A low detection level Western blot technique was used to increase the sensitivity of the experiment and to compare pgp expression in WHV-infected nonneoplastic liver compared with HCC (Fig. 2). In all three animals, there was an increase in pgp expression in the tumor compared with normal, nonneoplastic tissue from the same animal, corresponding to HC019, HC037, and HC017, respectively. Pgp expression was not detectable in samples of nonneoplastic liver obtained from the same animal as the HCC sample. The degree of staining intensity did vary from animal to animal using the chemiluminescent detection system, which is probably because of variation between animals. A summary of the animals used in the experiments is presented in Table 1. Note that the use of quantitative Western blot analysis confirmed previous identification by the colorimetric analysis of a pgp in HC019 (Fig. 1, lane 2 compared with Fig. 2, lane 2).

In rats treated with the Solt Farber protocol for chemical carcinogenesis, pgp is increased in preneoplastic and neoplastic lesions of the liver.[31] To test whether or not a similar pattern might be observed in

TABLE 1. Woodchuck Liver Examined to Detect pgp Expression

Animal No.	Tissue	WHV	Sex	Western Blot	IHC	RT-PCR
HC019	HCC*	+	F	‡	‡	‡
HC019	Nontumor*	+	F	‡	‡	‡
HC018	HCC*	+	F	‡	‡	‡
HC032	HCC*	+	F	‡	‡	‡
HC037	HCC*	+	F	‡	‡	‡
HC037	Nontumor*	+	F	‡	‡	‡
HC017	HCC*	+	M	‡	‡	‡
HC017	Nontumor*	+	M	‡	‡	‡
HC075	HCC*	+	M	‡	‡	‡
HC028	HCC*	+	F	‡	‡	‡
HC015	HCC†	+	F	‡	‡	‡
HC025	Nontumor†	+	F	‡	‡	‡
HC060	Nontumor†	+	F	‡	‡	‡
HC070	Nontumor†	+	M	‡	‡	‡
HC064	Nontumor†	+	M	‡	‡	‡
HC042	Nontumor†	+	F	‡	‡	‡
HC071	Nontumor†	+	F	‡	‡	‡
HC066	Nontumor†	+	M	‡	‡	‡
HC075	Nontumor†	+	M	‡	‡	‡
HC072	Nontumor†	+	M	‡	‡	‡
HC086	Nontumor†	−	M	‡	‡	‡
CW1021	Nontumor†	−	M	‡	‡	‡
Total				8	19	6

NOTE. Composite of data collected for woodchuck pgp and MDR characterization. Biopsy samples were surgically obtained, therefore, the woodchucks were not sacrificed at the time of those experiments.

Abbreviation: IHC, immunohistochemistry.

* Samples taken from necropsy.

† Samples taken from biopsy.

‡ Test performed.

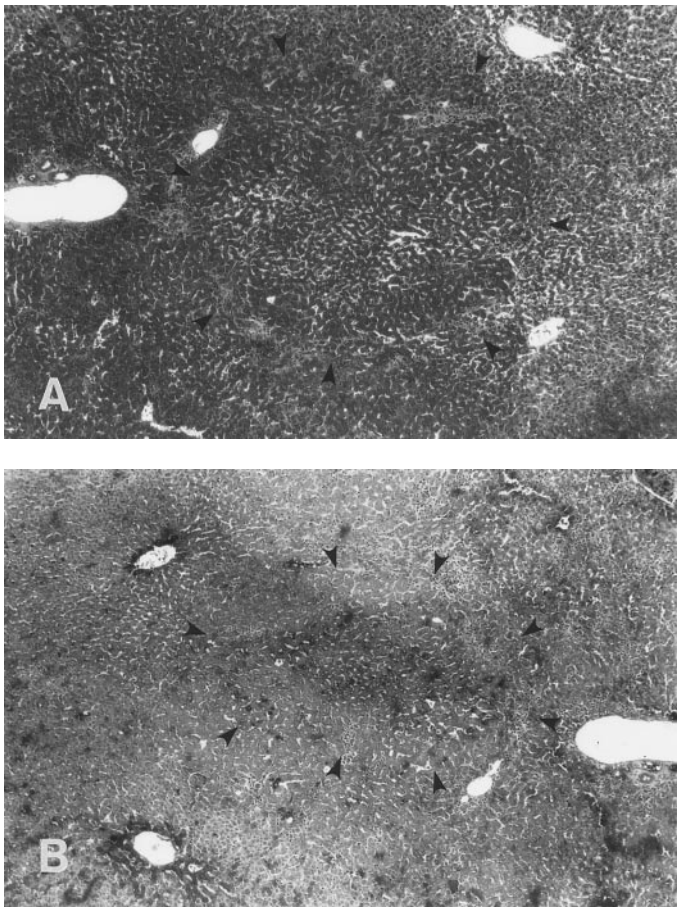

FIG. 3. (A) A hematoxylin and eosin–stained section of woodchuck liver with a preneoplastic basophilic focus, (B) serial section of the basophilic focus stained with JSB-1. There was focal intensification of staining for pgp in the center of the lesion as indicated by the arrows. Some centrilobular hepatocytes stained for pgp; otherwise, the nonneoplastic area of the liver did not stain for pgp. (Original magnification ×200.)

a virally transformed rodent model for hepatocarcinogenesis, woodchuck liver samples were stained immunohistochemically to compare nonneoplastic liver, preneoplastic, and neoplastic lesions on the basis of pgp content and distribution. Multiple sections from different parts of the same liver were obtained for comparison. A total of six animals were used for these studies. Sections of liver that contained basophilic foci, based on review of hematoxylin-eosin–stained sections, were serially sectioned and stained immunohistochemically for pgp. These experiments were performed to determine whether or not pgp could be detected in preneoplastic lesions. A panel of three monoclonal antibodies (JSB-1, C219, and C494) were used. Immunolocalization of pgp was identical for all antibodies. Stained foci (preneoplastic lesions) were observed in three of six animals. From the six animals, 20 basophilic foci were observed, and seven were positive for pgp. Figure 3A is a hematoxylin-eosin–stained basophilic focus and Fig. 3B is a serial section of the same tissue block stained with JSB-1. This particular focus was stained

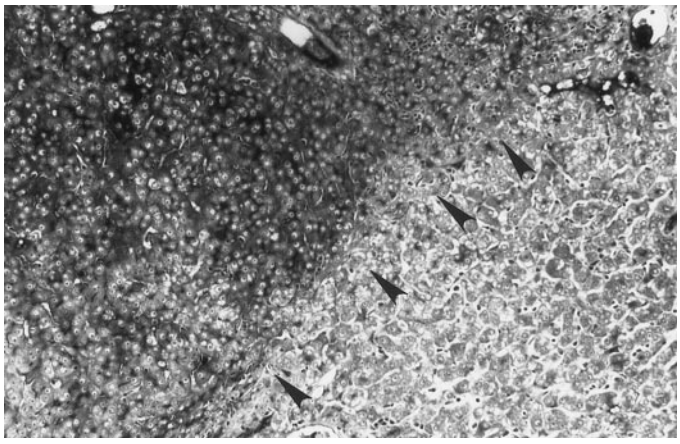

FIG. 4. Immunohistochemistry of woodchuck HCC using JSB-1. A ridge of compression demarked the interface between the HCC and nonneoplastic liver. (Original magnification ×200.)

with all three antibodies. Staining of the cytoplasm of centrilobular hepatocytes was occasionally observed (Fig. 3B). These areas were histologically normal and not part of a HCC. Staining was not typically observed in WHV-infected or WHV-uninfected liver (data not shown), although in one animal pgp was consistently observed in tissue adjacent to the HCC (Fig. 4, right of arrows).

In most HCC, there was a marked increase in pgp expression (Fig. 4). Several tissue sections were ana-

TABLE 2. pgp Expression in Normal Liver, Preneoplastic Foci, and HCC

Animal No.	Source	Normal Liver	Foci	HCC
	WHV–			
HC086	Biopsy	—	NA	NA
CW1021	Biopsy	—	NA	NA
	WHV+			
HC018	Necropsy	—	2/2	4/4
HC037	Necropsy	—	3/6	2/5
HC075	Necropsy	—	0/5	2/4
HC017	Necropsy	—	2/3	2/3
HC019	Necropsy	—	0/2	1/3
HC032	Necropsy	—	0/2	1/3
HC025	Biopsy	—	—	—
HC060	Biopsy	—	—	—
HC070	Biopsy	—	—	—
HC064	Biopsy	—	—	—
HC042	Biopsy	—	—	—
HC071	Biopsy	—	—	—
HC066	Biopsy	—	—	—
HC075	Biopsy	—	—	—
HC072	Biopsy	—	—	—
Total			7/20	12/22

NOTE. Surgical biopsy specimens and HCC (obtained at necropsy) were stained for pgp expression using JSB-1.

Abbreviations:— no staining detected; NA, not applicable, because neither foci or HCC were observed in WHV– (uninfected) woodchucks.

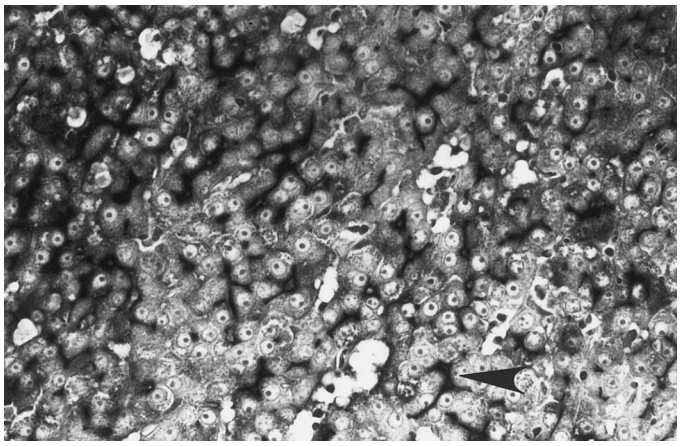

FIG. 5. High-power magnification of pgp staining within a HCC stained with JSB-1 as the primary antibody. Note both the intense staining at the bile canaliculi (arrow) and the diffuse cytoplasmic staining in some hepatocytes. (Original magnification ×400.)

lyzed from each animal. At least one HCC from each of the six animals tested had increased pgp expression; however, not all HCC were positive when different HCC samples were obtained from each animal. As summarized in Table 2, 12 of 22 HCC were positive for pgp. Whether the tumors were metastasis from one tumor or were independent tumors could not be determined. Neoplastic hepatocytes were characterized by diffuse cytoplasmic staining as well as prominent canalicular staining of pgp (Fig. 5).

Because this was the first report of pgp expression in woodchuck HCC, messenger RNA (mRNA) expression of the MDR gene was investigated to further characterize pgp expression. Three categories of samples were evaluated: HCC,WHV+ (which represented virus-infected liver from nontumor-bearing woodchucks), and WHV− (liver samples obtained from animals that were not virus carriers). Virus-infected and uninfected animals did not have HCC and were considered nontumorous samples. All of the samples analyzed by semiquantitative RT-PCR (HCC, WHV+, and WHV−) yielded an appropriately sized product, predicted on the basis of the sequence for human MDR1 (Fig. 6). The detection of a single PCR product, identical in migration to the HepG2 RNA, suggests that the woodchuck codes for mRNA from a MDR1-like gene, because the primers were chosen based on their MDR1 specificity in humans. When the data was pooled, there was no clear difference in levels of mRNA when HCC, WHV+, and WHV− expression levels were compared (Fig. 7A-C). On the contrary, HC018 (tumor) compared with HC060 (normal) illustrated a modest increase in tumor mRNA (Fig. 7B). These samples were run in at the same time, and prepared from the same master mix, therefore all of the reagents were the same; the only variable was the sample RNA.

It is possible that the primers used coincidently produced a PCR fragment that was not MDR, therefore an oligonucleotide internal to the PCR primers was used for hybridization to further define the MDR1 sequence homology. Selection of the oligonucleotide based upon a conserved region of the human MDR1 gene. The PCR products were transferred to nitrocellulose and hybridized. Hybridization was observed in the four HCC and four WHV+ nonneoplastic livers tested (Fig. 8).

DISCUSSION

The overexpression of MDR genes and their gene products is common during hepatocarcinogenesis. Mdr1/mdr3 overexpression has been reported in tumors spontaneously arising in C3H/HeN mice[31] and in transgenic mice containing the hepatitis B virus envelope gene.[32] In addition, C57BL/6N and B6C3F1 mice treated by two different chemical carcinogenic regimens overexpress mdr3 during hepatic tumor development.[31] In a multistep model of carcinogenesis, mice treated which chemical carcinogens overexpress pgp as a late event relative to other markers of progression such as γ-glutamyl transpeptidase and glutathione-*S*-transferase π.[33] Taken together, the overexpression of MDR may be an example of common molecular processes that occur during hepatocarcinogenesis, irre-

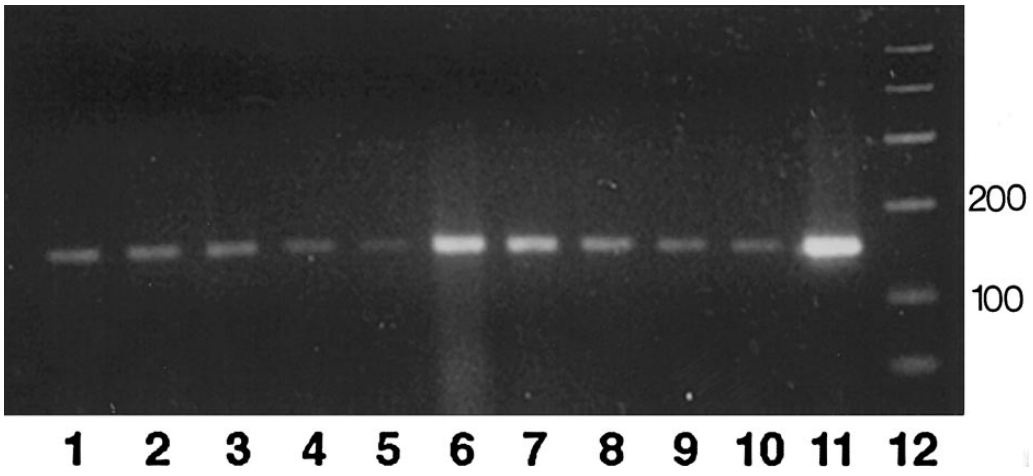

FIG. 6. Semiquantitative RT-PCR for MDR expression in nonneoplastic WHV+ liver compared to HCC. Serial dilutions of RNA represent 25 ng, 12.5 ng, 6.25 ng, 3.125 ng, and 1.56 ng in lanes 1-5 (WHV+, HC060) and lanes 6-10 (HCC, HC018). HepG2 cells (25 ng) were run as a positive control (lane 11). The PCR products migrated between 100 and 200 base pairs according to their migration compared with molecular-weight standards (lane 12).

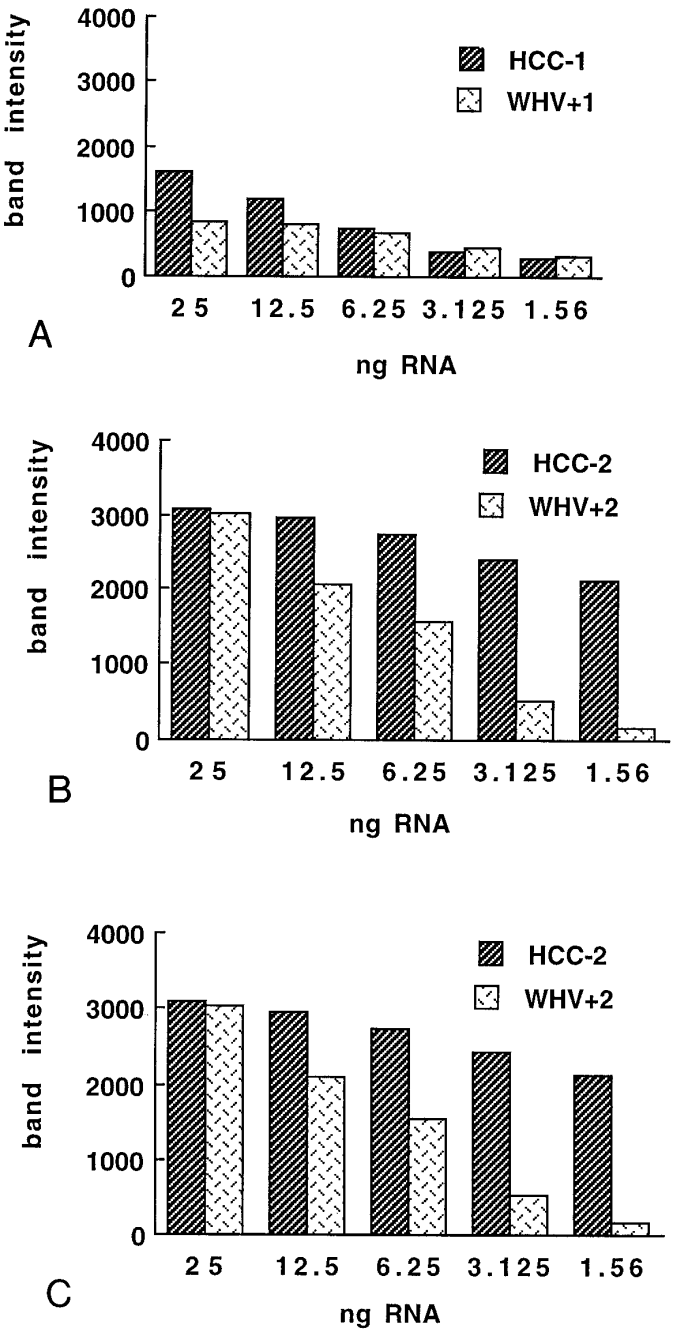

FIG. 7. Summary of results from semiquantitative RT-PCR for HCC, WHV+, and WHV− liver amplified with MDR1 primers. Two different sets of HCC and WHV+ samples were run in parallel (A and B), and two individual samples were also analyzed from WHV− woodchucks (C). HCC-1, HCC-2, WHV+1, WHV + 2, WHV − 1, and WHV − 2 corresponded to woodchucks HC0109, HC018, HC025, HC060, HC086, and CW1021, respectively.

spective of cause.[34] Our data support this hypothesis, because we have now shown that the woodchuck, like other rodent models for hepatocarcinogenesis, overexpress pgp in some preneoplastic and neoplastic lesions. We contend that the protein identified in these lesions is a member of the pgp family based upon Western blot specificity, known cross-reactivity of the antibodies used with other rodent pgps, and the highly conserved nature of these proteins phylogenetically.

The authenticity of the pgp was further substantiated by identification of a MDR1-like gene in the woodchuck (Figs. 6 and 7). This sequence similarity was anticipated because human MDR1 probes have been used to measure MDR expression in other rodent cells.[35] Levels of mRNA for MDR have been shown to increase during liver regeneration following partial hepatectomy[36] and in tumors induced with chemical carcinogens.[37] In the semiquantitative RT-PCR technique used to evaluate mRNA levels in the woodchuck, there appeared to be a trend toward more mRNA in the HCC compared with WHV+ liver in one sample set (tumor vs. normal). Overall, there was no measurable difference in mRNA expression when the data was evaluated collectively. This may be because a limited number of samples were analyzed, or it may be because of a difference in the sensitivity of the techniques employed. Regulation of woodchuck MDR may also be different from rats and mice.

Whether or not the woodchuck pgp functions like MDR1 or MDR2 remains to be determined. Although the Mab cross-reacted with the woodchuck protein by Western blot and immunohistochemistry analyses, the selectivity of these antibodies in the woodchuck is unknown at this time. In mice, it is believed that JSB-1 binds only to the gene product of MDR1, which is activated during drug resistance, but we cannot extrapolate this information to conclude that the protein detected in the woodchuck functions in drug resistance with certainty.

Nonspecific binding was a problem when JSB-1 was used in combination with the enhanced chemiluminescent detection system. Using the colorimetric procedure for pgp detection, nonspecific binding was not as great as when the chemiluminescent detection method was used. We believe that it is likely because of differences in the sensitivity of the assays. It could also be a result of nonspecific binding of the secondary antibody to woodchuck proteins. Several different blocking procedures were used to minimize nonspecific binding, the best one was an overnight blocking step in 10% nonfat dried milk dissolved in PBS. To our dissatisfaction, as

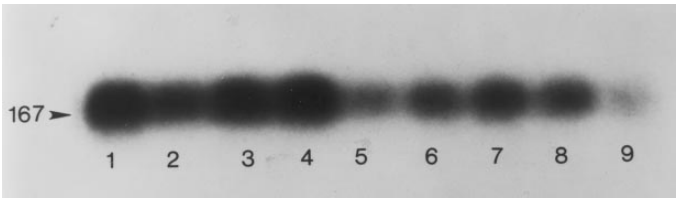

FIG. 8. Southern blot analysis of PCR products hybridized with a MDR1 specific probe internal to the primers used to amplify the cDNA. Each lane represents cDNA from 1 μg of reverse-transcribed total RNA. The MDR probe hybridized to HCC from HC019, HC018, HC032, and HC037, run in lanes 1-4, respectively, and WHV+ from HC025, HC060, HC070, and HC064, run in lanes 5-8, respectively. Canine kidney (1 μg) was run as a positive control (lane 9).

many as six proteins were nonspecifically detected in woodchuck HCC. The use of antibodies across species can commonly result in such a dilemma.

The highly conserved nature of MDR and its gene products, pgp, enabled us to investigate the expression of this drug resistance marker in the woodchuck using mouse and human probes. We have shown an increased expression of pgp in woodchuck HCC, and detected MDR1-like mRNA in HCC, WHV+, and WHV− liver samples. Efforts are currently underway to elucidate the functional role of the woodchuck pgp identified by these studies. If the pgp identified does function to transport anticancer drugs, the woodchuck model will clearly be a useful tool for studying the acquisition resistance during viral carcinogenesis. This may open new avenues of inquiry into the role of virus-MDR interactions and the development of innovative therapeutic modalities.

REFERENCES

1. Gottesman M. How cancer cells evade chemotherapy: sixteenth Richard and Hilda Rosenthal Foundation Award lecture. Cancer Res 1993;53:747-754.
2. Gottesman M, Pastan I. The multidrug transporter, a double edge sword. J Biol Chem 1988;263:12163-12166.
3. Croop J, Gros P, Housman D. Genetics of multidrug resistance. J Clin Invest 1988;81:1303-1309.
4. Endicott J, Ling V. The biochemistry of p-glycoprotein mediated multidrug resistance. Annu Rev Biochem 1989;58:137-171.
5. Gottesman M. Multidrug resistance during chemical carcinogenesis: a mechanism revealed. J Natl Cancer Inst 1988;80:1352-1353.
6. Lightdale C, Ikram H, Pinsky C. Primary hepatocellular carcinoma with hepatitis B antigenemia: effects of chemotherapy. Cancer 1980;46:1117-1122.
7. Beasley P. Hepatitis B virus as the etiologic agent in hepatocellular carcinoma-epidemiological considerations. HEPATOLOGY 1982;2:215-255.
8. Park J, Lee S, Hong I, Kim H, Lim K, Choe K, Kim W, et al. MDR1 gene expression: its effect on drug resistance to doxorubicin in hepatocellular carcinoma cell lines. J Natl Cancer Inst 1994;86:700-705.
9. Goldstein L, Galski H, Fojo A, Willingham M, Lai S, Gasdar A, Pirker R, et al. Expression of multidrug resistance gene in human cancers. J Natl Cancer Inst 1988;81:116-124.
10. Itsubo M, Ishikawa T, Toda G, Tanaka M. Immunohistochemical study of expression and cellular localization of the multidrug resistance gene product p-glycoprotein in primary liver carcinoma. Cancer 1994;73:298-303.
11. Rothenberg M, Ling V. Multidrug resistance: Molecular biology and clinical relevance. J Natl Cancer Inst 1989;81:907-910.
12. Yeh GC, Lopeczynska J, Poore CM, Phang JM. The new functional role for p-glycoprotein: efflux pump for benzo(a)pyrene in human breast cancer MCF-7 cells. Cancer Res 1992;52:6692-6695.
13. Kurelec B, Pivcevic B. Evidence for a multixenobiotic resistance mechanism in the mussel mytilus galloprovincialis. Aquatic Tox 1991;19:291-302.
14. Smit J, Schinkel A, Oude-Elferink R, Groen A, Wadenaar B, von Deemter L, Mol C. Homozygous disruption of murine mdr2 p-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 1993;75:451-462.
15. Gros P, Neriah Y, Croop J, Houseman J. Isolation and expression of a complementary DNA that confers multidrug resistance. Nature 1986;323:728-731.
16. DeVault A, Gros P. Two members of the mouse mdr gene family confer multidrug resistance with overlapping but distinct drug specificities. Mol Cell Biol 1990;10:1652-1663.
17. zur Hausen H. Viruses in human cancer. Science 1991;254:1167-1173.
18. Gerin JL, Cote PJ, Korba BE, Tennant BC. Hepadnavirus-induced liver cancer in woodchucks. Cancer Detect Prev 1989;14:227-229.
19. Galibert F, Chen TN, Mandart E. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: comparison with the hepatitis B virus sequence. J Virol 1982;41:51-65.
20. Cote PJ, Engle RE, Langer CA, Ponzetto A, Gerin JL. Antigenic analysis of woodchuck hepatitis virus surface antigen with site specific radioimmunoassays. J Virol 1984;49:701-708.
21. Ponzetto A, Cote PJ, Ford EC, Engle R, Cicmanec J, Shapiro M, Purcell R. Radioimmunoassay and characterization of woodchuck hepatitis virus core antigen and antibody. Virus Res 1985;2:301-315.
22. Cote PJ, Sharpiro M, Engle RE, Popper H, Purcell RH, Gerin JL. Protection of chimpanzees from type B hepatitis by immunization with woodchuck hepatitis virus surface antigen. J Virol 1986;60:895-901.
23. Van der Valk P, van Kalken C, Ketelaars H. Distribution of resistance-associated P-glycoprotein in normal and neoplastic human tissues. Ann Oncol 1990;1:56-64.
24. Weinstein R, Grogan T, Kuszak J, Jakate S, Kluskens L, Coon J. Multidrug resistance gene product (P-glycoprotein) in normal tissue and tumors. In: Clark G, ed. Advances in pathology and laboratory medicine. New York: Academic Press, 1991:207-234.
25. Lieberman D, Reithmeier A, Ling V, Charuk J, Goldberg H, Skorecki K. Identification of P-glycoprotein in renal brush border membranes. Biochem Biophys Res Commun 1989;162:244-252.
26. Bell D, Gerlach J, Kartner N, Buick R, Ling V. Detection of P-glycoprotein in ovarian cancer: a molecular marker associated with multidrug resistance. J Clin Oncol 1985;3:311-315.
27. Georges E, Sharom F, Ling V. Multidrug resistance and chemosensitization: therapeutic implications for cancer chemotherapy. In: McArdell T, ed. Advances in pharmacology. New York: Academic Press, 1990:185-221.
28. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
29. Noonan K, Beck C, Holzmayer T, Chin J, Wunder J, Andrulis I, Gazdar A, et al. Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. Proc Natl Acad Sci U S A 1990;87:7160-7164.
30. Chen C, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, Roninson IB. Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug resistant human cells. Cell 1986;47:381-389.
31. Teeter LD, Becker F, Chisari FV, Li D, Kuo MT. Overexpression of the multidrug resistance gene mdr3 in spontaneous and chemically induced mouse hepatocellular carcinomas. Mol Cell Biol 1990;10:5728-5735.
32. Kuo MT, Zhao JY, Teeter LD, Ikeguchi M, Chisari FV. Activation of multidrug resistance (P-glycoprotein) mdr3/mdr1a gene during the development of hepatocellular carcinoma in hepatitis B virus transgenic mice. Cell Growth Differ 1992;3:531-540.
33. Kuo MT. Expresson of multidrug-resistance (P-glycoprotein) genes in liver cancers: a molecular example of the convergence theory of hepatocarcinogenesis. Mol Carcinog 1993;7:73-75.
34. Bradley G, Sharma R, Rajalakshmi S, Ling V. P-glycoprotein expression during tumor progression in rat liver. Cancer Res 1992;52:5154-5161.
35. Chin K, Chauhan S, Pastan I, Gottesman M. Regulation of mdr RNA levels in response to cytotoxic drugs in rodent cells. Cell Growth Differ 1990;1:361-365.
36. Nakatsukasa H, Everts R, Burt R, Nagy P, Thorgeirsson S. Cellular pattern of multidrug resistance gene expression during chemical hepatocarcinogenesis in the rat. Mol Carcinog 1992;6:190-198.
37. Burt R, Thorgeirsson S. Coinduction of MDR1 multidrug resistance and cytochrome p-450 genes in rat liver by xenobiotics. J Natl Cancer Inst 1988;80:1383-1386.