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Duck hepatitis B virus DNA in liver and serum of Chinese ducks: Integration of viral DNA in a hepatocellular carcinoma

(viral replication/hybridization/neoplasm)

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ABSTRACT **The presence of duck hepatitis B virus (DHBV) DNA in liver and serum and its state (integrated vs. free) were studied in 23 ducks from Chi-tung county in China by spot hybridization and Southern blot hybridization, respectively. In 16 of 23 (70%), DHBV DNA was detected in serum and/or in liver tissue. These infected ducks showed a variety of pathological changes including advanced chronic disease in the liver. In contrast, none of the virus-negative ducks had advanced hepatic changes. One DHBV DNA-seropositive duck had a large hepatocellular carcinoma. Southern blot analysis demonstrated integrated DHBV DNA in neoplastic tissue and abundant episomal DHBV DNA in non-neoplastic tissue of the liver. In one noninfected duck with a small adenoma, no viral DNA was detected in tumor or non-neoplastic tissue. The detection of integrated DHBV DNA in hepatocellular carcinoma suggests that DHBV behaves like human and woodchuck hepatitis viruses in relation to chronic liver disease and hepatocarcinogenesis.**

Hepatitis B virus infection is closely related to acute and chronic liver disease and hepatocellular carcinoma in man. However, the study of pathogenesis of human liver disease is hampered by ethical considerations, which preclude many experiments, and lack of *in vitro* techniques for virus propagation. With the recently discovered woodchuck hepatitis virus (1), ground squirrel hepatitis virus (2), and duck hepatitis B virus (DHBV) (3), ways have been provided to study the pathogenesis of liver disease in lower animals under controlled conditions.

A variety of liver diseases, including hepatocellular carcinoma, have been observed in ducks from Chi-tung county in China (4), and the carrier state has been established in Japanese ducklings by postnatal inoculation with DHBV-positive sera (5).

Although integration of viral DNA into genomic DNA of hepatocellular carcinoma tissue in man (6–9) and in woodchucks (10, 11) has been detected by use of the Southern blot technique, the same has not been found in hepatocellular carcinoma of the duck. We now report our study on DHBV DNA in serum and liver specimens of 23 ducks from Chi-tung county in China.

MATERIALS AND METHODS

Detection of DHBV DNA in Serum. A hybridization spot test was performed on 200-μl aliquots of serum. Each aliquot was added to 1 ml of 20 mM Tris Cl, pH 8.0/150 mM NaCl/10 mM EDTA/0.2% $NaDodSO_4$ containing 10 mg of Pronase and 10 μg of wheat germ ribosomal RNA. After incubation at 37°C for 2 hr, the solution was extracted with an equal volume of phenol equilibrated with 20 mM Tris Cl, pH 8.0/150 mM NaCl/10 mM EDTA. After centrifugation, the aqueous phase was removed, and nucleic acids were precipitated by addition of 2 volumes of absolute ethanol, washed with ethanol, and dried. The pellet was resuspended in 20 μl of 5 mM Tris Cl, pH 7.4/1 mM EDTA, and 10 μl of the suspension was spotted on nitrocellulose paper. Hybridization with a ^{32}P-labeled DHBV DNA probe and autoradiography were as described (5).

Detection of DHBV DNA in Liver. Liver tissue (200 mg) was homogenized in 5 ml of ice-cold 0.01 M Tris Cl, pH 7.4/0.01 M EDTA, followed by addition of 5 ml of 0.2 M NaCl/0.02 M Tris Cl, pH 7.4/2 mM EDTA/1% $NaDodSO_4$ containing 1 mg of Pronase per ml and incubation for 2 hr at 37°C. The nucleic acids were deproteinized by two extractions with Tris-buffered phenol/chloroform (pH 7.4), followed by one extraction with chloroform and precipitation with absolute ethanol. After electrophoresis in a horizontal agarose slab gel, DNA was transferred to nitrocellulose filter paper by the method of Southern (12) as modified by Wahl *et al.* (13). The filter paper was baked dry and hybridized with ^{32}P-labeled DHBV DNA as described (5).

For quantitation of DHBV DNA in tissue, 10 μg of DNA extract from the liver of each animal and various amounts (100, 50, 25, 5, 1, 0.5, and 0.1 pg) of cloned DHBV DNA as standards were spotted on a nitrocellulose paper. Autoradiographic spots were analyzed with a Sakura PDS 15 densitometer (Sakura, Tokyo), and the hybridization signals were compared with those for the cloned DHBV DNA standards. DHBV DNA in serum was quantitated similarly.

Ducks. Liver tissue and serum were obtained from 23 ducks (1–3 years old) from Chi-tung county in China. They were purchased from a duck farm. This was a different batch of Chi-tung ducks from the one used in a previous study (4). Liver tissue was quick-frozen and kept at −80°C until use. Of the 23 ducks, 4 were White Pekin, and the remainder were "Chinese" ducks (14).

Histological Study of Duck Livers. Liver tissue was fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin/eosin, with periodic acid/Schiff reagent, or by the azan method (Heidenhain's modification of Mallory's triple stain) for collagen fibers. Histological classification was as follows. Nonspecific changes (NSC): no or few inflammatory cells in portal areas with no hepatocellular degeneration and no necrosis in the parenchyma. Chronic persistent hepatitis (CPH)-like: minimal to moderate mononuclear cells without disruption of limiting plates, minimal hepatocellular degeneration, and some increase in number of sinusoidal cells (Fig. 1*A*). Chronic active hepatitis (CAH)-like: moderate to marked portal mononuclear cell infiltrates with disruption of

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Abbreviations: DHBV, duck hepatitis B virus; NSC, nonspecific changes; CAH, chronic active hepatitis; CPH, chronic persistent hepatitis.

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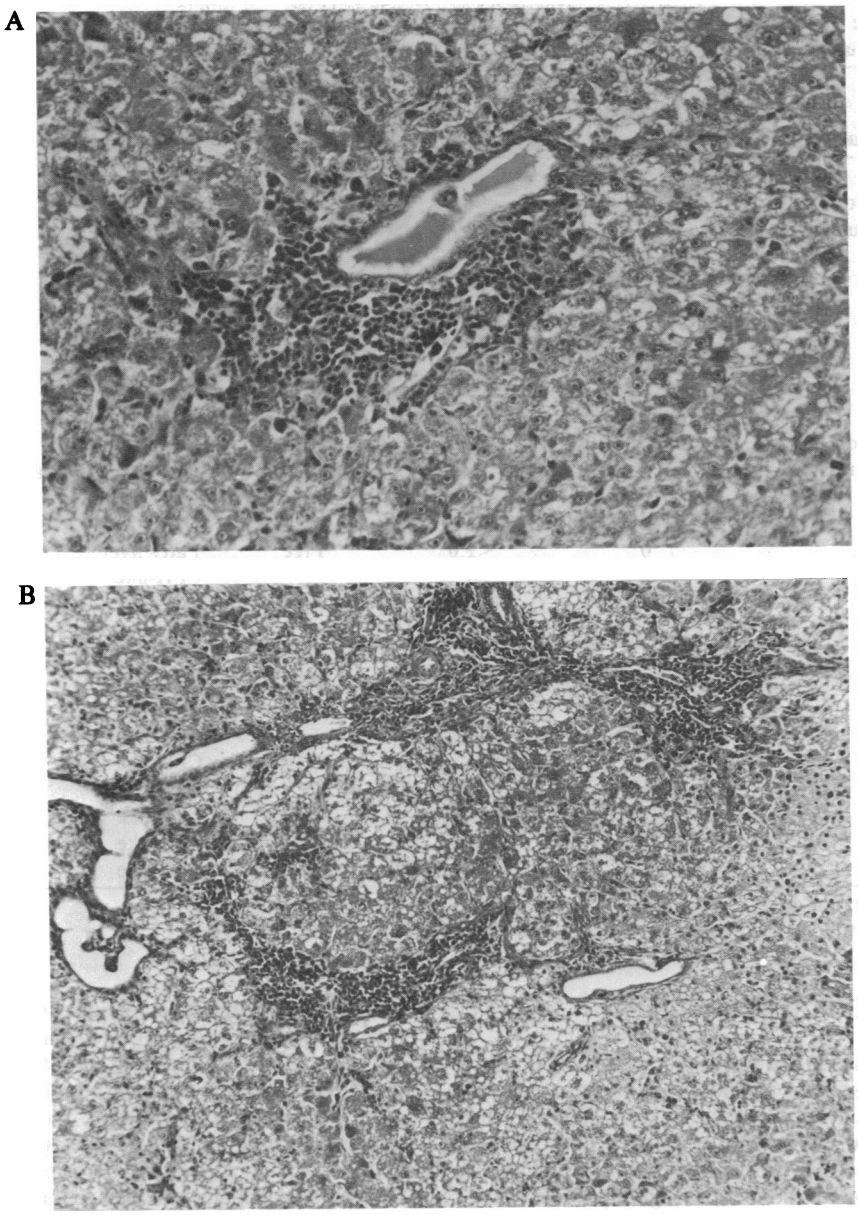

FIG. 1. (*A*) Photomicrograph of the liver of duck no. 19, showing CPH-like changes with minimal portal infiltrates and intact limiting plates. (Hematoxylin/eosin; ×180.) (*B*) Photomicrograph of the liver of duck no. 17, showing CAH-like changes with portal and parenchymal infiltrates and significant fibrosis. (Hematoxylin/eosin; ×72.)

limiting plates, diffuse hepatocellular degeneration and parenchymal infiltrates, and fibrous septa radiating into the parenchyma (Fig. 1*B*). Regenerative nodules and prominent ductule formation could be seen.

RESULTS

Viral DNA. Nine of 22 (41%) ducks were positive for DHBV DNA by serum testing alone, whereas 16 of 23 (70%) were positive for DHBV DNA when both liver and serum specimens were tested (Table 1). Of the 4 White Pekin ducks, only no. 4 had a trace amount of viral DNA in liver tissue. In contrast, 15 of 19 (79%) "Chinese" ducks were infected (Table 1).

Histological Characteristics of the Liver. Histological changes in the 23 ducks included fatty liver in 3, multiple cysts in 1, CPH-like changes in 9, and CAH-like changes in 6 (Table 1). Advanced liver disease (CAH-like changes) was found only in the infected ducks (6 of 16) and not in noninfected ducks (0 of 7).

Neoplastic lesions were found in 2 ducks: no. 1 had a whitish nodule 2.5 cm in diameter and no. 8 had a massive tumor that involved more than half of the liver (Fig. 2*A*). Microscopically, the former appeared to be a bile duct adenoma, probably benign (Fig. 2*B*), and the latter showed macrotrabecular arrangements of malignant cells with eosinophilic cytoplasm and prominent nucleoli, the characteristic features of hepatocellular carcinoma in man (Fig. 2 *C* and *D*).

Viral DNA in Tumor Tissue. In duck no. 1 (White Pekin), DHBV DNA was not detected in serum, in the adenoma, or in non-neoplastic liver tissue. In duck no. 8 (Chinese duck), DHBV DNA was detected in both serum and liver tissue. Southern blot analysis of the tumor revealed a signal at very high molecular weight with uncut DNA (Fig. 3, lane 1) and several discrete bands, at molecular weights corresponding to more than 3 kilobase pairs, after enzyme digestions (Fig. 3, lanes 2 and 3), suggesting specific-site integration of viral DNA into cellular DNA. In non-neoplastic tissue, a large amount of free viral DNA was detected (Fig. 3, lanes 4–6).

Table 1. DHBV DNA in the liver and serum and histological characteristics of the liver in 23 Chi-tung ducks

Duck no.	Serum DHBV DNA, pg/100 μl	Liver DHBV DNA, pg/μg of DNA	State of DHBV DNA in liver*	Histological classification
		White Pekin ducks		
1 Non-tumor	0	0	–	CPH-like
Tumor			–	Adenoma
2	0	0	–	Fatty liver
3	0	0	–	CPH-like
4	0	<1.0	Free	Cyst
		Chinese ducks		
5	0	0	–	NSC
6	190	1520	Free	CAH-like
7	0	1	Free	Fatty liver
8 Non-tumor	30	600	Free	CAH-like
Tumor			Integrated	Hepatocellular carcinoma
9	0	0	–	NSC
10	40	1050	Free	NSC
11	0	<1.0	Free	Fatty liver
12	60	0	–	CPH-like
13	0	470	Free	CAH-like
14	0	0	–	CPH-like
15	0	70	Free	CAH-like
16	50	ND	ND	CAH-like
17	10	200	Free	CAH-like
18	0	0	–	CPH-like
19	40	350	Free	CPH-like
20	5	0	–	NSC
21	ND	250	Free	CPH-like
22	70	500	Free	CPH-like
23	0	<1.0	Free	CPH-like

ND, not done.
*Determined by Southern blot hybridization.

DISCUSSION

A variety of liver diseases in the ducks from Chi-tung county in China have been described previously (4). In that study, only the presence of DHBV in serum was examined; the molecular state of viral DNA in liver tissue could not be determined because no fresh frozen liver was available. In the current study, we found a somewhat higher prevalence of infection (70%) compared to that reported in ref. 4 (50%) and other studies (55%; ref. 15) of Chi-tung ducks. Viral DNA was more often detected in liver than in serum. However, in two ducks (nos. 12 and 20), viral DNA was detected only in serum. Although it is tempting to speculate that viral DNA is derived from viral replication in extrahepatic tissues (3, 16, 17), we do not know to what extent the extrahepatic replication of the virus contributes to the circulating virions.

It was particularly noteworthy that "Chinese" ducks had a very high prevalence of infection (79%), and frequent liver disease (Table 1). In contrast, viral DNA was detected in only 1 of 4 White Pekin ducks, and none of these ducks showed CAH-like changes in the liver. Marion *et al.* suggested that differences in duck breed might be an important factor in the development of liver disease (15). Whether Chinese ducks are more susceptible to liver disease with DHBV infection than the White Pekin variety has to be determined with a greater number of ducks and by experimental transmission. According to the zoological description of the Chinese duck (14), these ducks were reared in China for many centuries and were herded in hundreds to fields where they fed on weeds, wasted rice, wild seeds, and water insects. It is conceivable that the liver disease in Chi-tung is related to other environmental factors, despite the very high prevalence of infection with DHBV. Since not all ducks with liver disease were infected (4, 15), and integrated viral DNA was not found in two neoplasms of the liver (hepatocellular carcinoma and malignant epithelial tumor), the role of DHBV infection in the pathogenesis of liver disease, particularly of liver cancer, among Chi-tung ducks was questioned recently (15).

In the current study, only the infected ducks had progressive morphological changes similar to those associated with chronic active hepatitis in man. Although the noninfected ducks showed mild inflammatory infiltrates that were limited in the portal areas, no advanced liver disease with prominent fibrosis and distortion of architecture was found. Furthermore, we studied two different types of liver tumors by Southern blot hybridization. Liver tissue was kept at −80°C until use, and ethidium bromide staining revealed that the nucleic acids extracted from the liver and neoplastic tissue were not degraded during preparation. One noninfected White Pekin duck had a bile duct adenoma with no detectable DHBV DNA. In contrast, one infected Chinese duck had a massive liver tumor which was typical hepatocellular carcinoma. Southern blot hybridization revealed integration of viral DNA into genomic DNA of the tumor, and abundant episomal DHBV DNA was detected in non-neoplastic tissue of the same liver (Fig. 3). This pattern has been reported often in human and woodchuck hepatocellular carcinoma (6–11). Beside the current study, we have performed Southern blot hybridization analysis of nucleic acids from more than 100 infected duck livers without tumor, including liver of DHBV-infected Japanese ducks (5), but the integration pattern has never been observed (data not shown).

Among four hepatitis B virus-like viruses, viral DNA integration into genomic DNA of hepatocellular carcinoma had been documented before only in human and woodchuck infection (6–11). This report of the integration of DNA of a

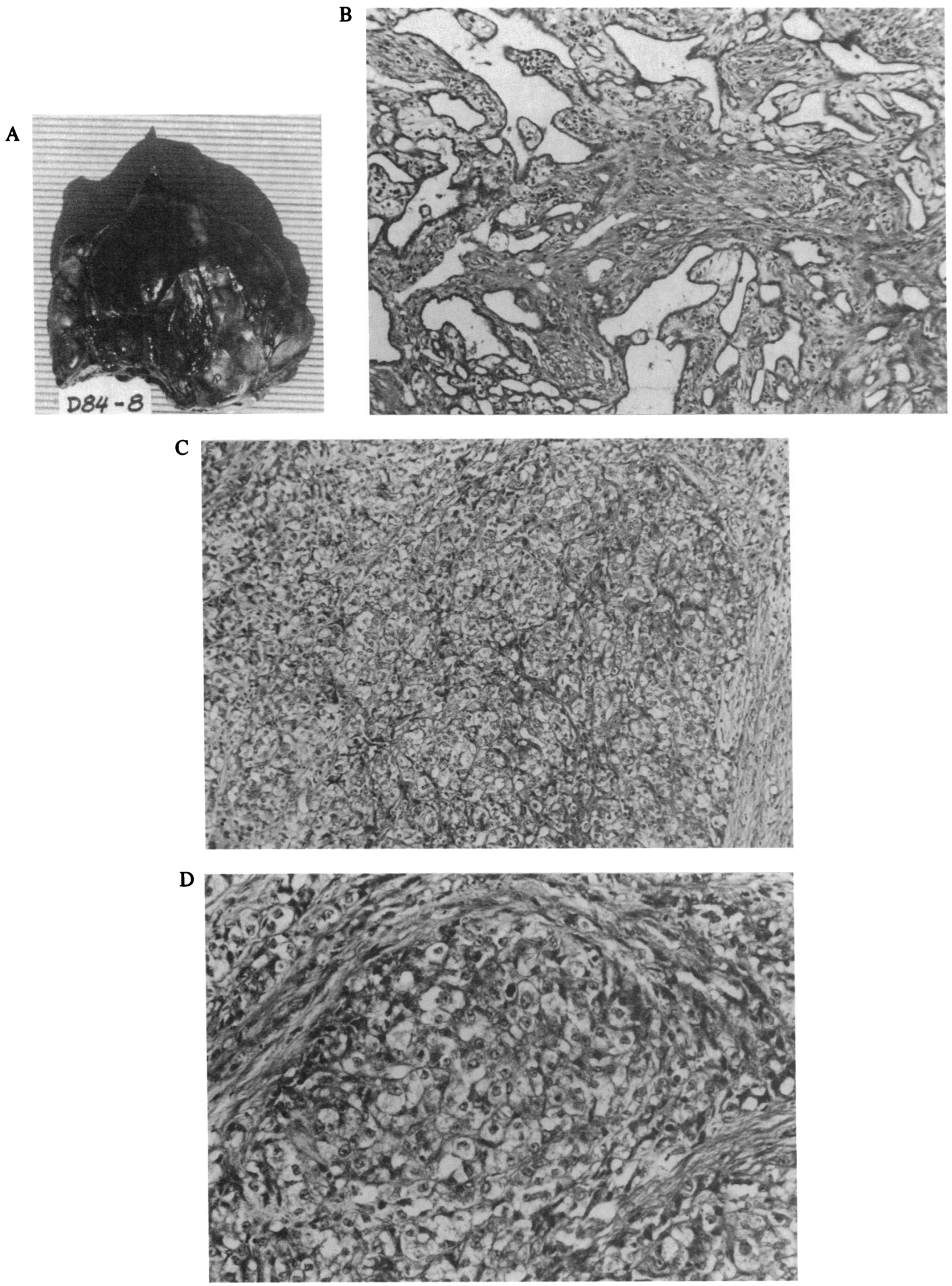

FIG. 2. (*A*) Massive liver tumor in one Chinese duck (no. 8). Non-neoplastic portion of the liver showing relatively smooth surface (noncirrhotic). (*B*) Photomicrograph of a small nodule of the liver in duck no. 1, showing adenoma with bile duct-like structures. (×90.) (*C*) Photomicrograph of the liver tumor in duck no. 8, showing macrotrabecular hepatocellular carcinoma. (×90.) (*D*) Close-up of the malignant cells, with prominent nucleoli and eosinophilic cytoplasm. (×180.) (Hematoxylin/eosin stain in *B–D*.)

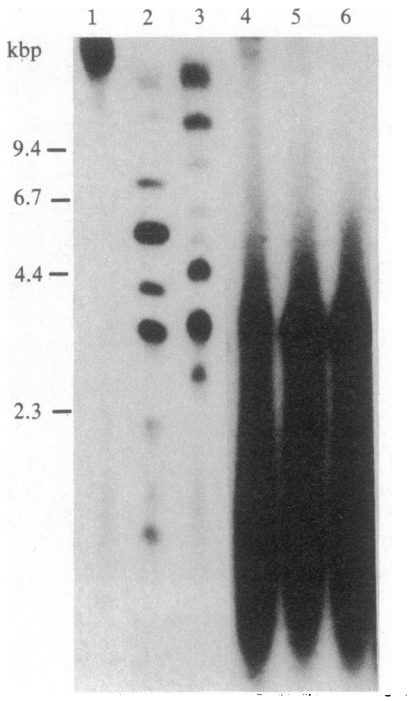

FIG. 3. Southern blot hybridization of DNA from hepatocellular carcinoma (lanes 1–3) and non-neoplastic liver (lanes 4–6) in duck no. 8. Each lane contained 20 μg of extracted nucleic acids that were undigested (lanes 1 and 4), *Eco*RI-digested (lanes 2 and 5), or *Hin*dIII-digested (lanes 3 and 6). kbp, Kilobase pairs.

third virus in hepatocellular carcinoma suggests similarities of DHBV to human and woodchuck hepatitis viruses in biological behavior.

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