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Evidence for increased *in vitro* recombination with insertion of human hepatitis B virus DNA

(chromosomal instability/chronic hepatitis/hepatocarcinogenesis)

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ABSTRACT **Chromosomal translocation, deletion, and inversion/duplication directly linked to hepatitis B virus (HBV) DNA integration occur frequently in host DNA of human hepatocellular carcinomas. To test the possible recombinogenic effect of HBV DNA, we have utilized an *in vitro* recombination assay. Fragments containing the region spanning DR1, which is believed to be the origin of viral replication and a preferred site in the viral genome for integration, increased the recombination events reproducibly in the presence of extracts from actively dividing cells (e.g., hepatocellular carcinoma) but not resting cells (e.g., normal liver). Moreover, in these extracts we have found a protein(s) that specifically binds to these HBV DNA fragments. These results support the notion that in some instances integrated HBV DNAs cause further genomic instability, possibly involving specific cellular protein(s). The fact that extracts from nondividing, normal liver did not increase recombination events suggests that genomic instability depends upon active cellular growth, a feature more commonly found subsequent to HBV-induced hepatocellular injury than in healthy liver. Our results offer an explanation for the high incidence of liver cancer that accompanies chronic hepatitis and add HBV to the list of agents that can cause genetic recombination.**

Persistent hepatitis B virus (HBV) infection is epidemiologically closely associated with the development of human hepatocellular carcinoma (HCC) (1). HBV has recently been shown to promote HCC in HBV carriers through several mechanisms. For example, a single case of HBV DNA integration has been reported into a retinoic acid receptor gene (2) and a cycline A gene (3), presumably illustrating a "cis-acting" promoter insertion mechanism. In several woodchuck HCCs, woodchuck hepatitis virus DNA has been found integrated into either of two members of the cellular *myc* oncogene family (c-*myc* or N-*myc*) (4). Nonetheless, the vast majority of HBV DNA integration cloned from human HCCs is not associated with human cellular oncogenes or a common cellular DNA sequence, and extensive studies have failed to detect mutations in *myc* oncogenes (4). Another mechanism, "trans"-activation of cellular growth-controlling genes by viral proteins, such as the X gene protein, has been suggested and it was recently reported that X gene induced liver tumors in transgenic mice (5). However, among HBV carriers, HCCs usually develop in patients with chronic liver disease, such as chronic active hepatitis or cirrhosis. In other words, hepatitis-related proliferative change, which is mainly sustained by repeating cycles of cell death and regeneration, mediated either immunologically or through virus infection, appears important for HBV hepatocarcinogenesis. Such a model has also been reported in hepatocarcinogenesis of transgenic mice expressing the large S gene of HBV (6). The accumulation of mutations, which are likely to occur during continuous cycles of cell division, may eventually transform some hepatocytes through a multistage process involving, among other things, the permanent loss of activity of genes that suppress cellular proliferation (7–12) [e.g., anti-oncogenes/tumor suppressor genes (13)]. In addition to these mechanisms, it has been proposed that HBV integration functions as a random mutagen promoting chromosomal defects in hepatocytes. This is supported by experiments showing HBV DNA integration at the sites of chromosomal translocation, large deletion, and inversion/duplication in host DNA of HCC (14–20). These examples have led to the notion that in some instances HBV DNA integration may enhance chromosomal instability. However, whether HBV DNA actually caused the chromosome defects as opposed to using them as an insertion point after they occurred is not known. In the present study, an *in vitro* system has been devised to test the hypothesis that genetic recombination could be mediated by HBV DNA. The experiments in this report provide evidence that HBV DNA promotes homologous recombination in target DNA at sites distant from the HBV insertion point.

MATERIALS AND METHODS

The design of our *in vitro* recombination assay is based on the idea that inserted DNAs will facilitate pairing of two plasmids in the presence of cell extracts that catalyze the reaction, and thereby enhance homologous recombination between two mutation sites within a drug-resistance gene, resulting in reversion from drug sensitivity to drug resistance (21).

DNA Preparation. Two mutant plasmid DNAs [tetracycline-sensitive (Tc^s) pBR322 derivatives, mut 1 and mut 2] were prepared by modifying the tetracycline gene (21). Mut 1 was obtained by treating the plasmid with *Eco*RV, which cut it near one end of the tetracycline-resistance gene, followed by digestion with BAL-31 to remove about 100 base pairs (bp), partial repair with Klenow fragment of DNA polymerase, and ligation with T4 DNA ligase. Mut 2 was obtained by digestion of the plasmid with *Xma* III, repairing the ends with the Klenow fragment, and ligation. This resulted in a 4-bp insertion at the *Xma* III site, which is near the other end of the tetracycline-resistance gene.

HBV DNAs. The HBV fragments, 5.4 and 3.2 kilobases (kb), to be tested in the *in vitro* recombination assay, were obtained from the integration at the site of a chromosome translocation in a HCC (14–16) [pBC4–5.4B (22, 23)] and wild-type HBV (24) (kindly provided by C. E. Rogler, Albert

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Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; DTT, dithiothreitol; Tc^r, tetracycline-resistant; Tc^s, tetracycline-sensitive; Amp^r, ampicillin-resistant.

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Einstein College of Medicine) by *Bam*HI and *Eco*RI digestion, respectively. The 1076-bp *Bgl* II (partial digestion), 440-bp *Bgl* II, and 196-bp *Bgl* II fragments were obtained from the pBC4–5.4B clone (Fig. 2). The 600-bp *Bgl* II, 450-bp *Eco*RI–*Bam*HI, and 300-bp *Eco*RI–*Bam*HI fragments were obtained from wild-type HBV (Fig. 2). These fragments were inserted into the *Eco*RI site of mut 1 and mut 2 in the same orientation by the blunt-end ligation method, and then these two mutant plasmids (mut 1 and mut 2) were incubated with reaction mixtures containing cell extracts from various tissues (Fig. 1).

Cell Extracts. Fresh tissues (Table 1) were homogenized with 5–10 volumes of buffer [20 mM Tris·HCl, pH 7.5/2 mM dithiothreitol (DTT)/1 mM EDTA/0.1 mM *p*-aminobenzamidine/2 μM spermidine/1 M NaCl], diluted with 7 volumes of the same buffer without NaCl, and centrifuged at 80,000 × *g* for 60 min to remove cellular DNA histones. The protein fraction was precipitated by addition of ammonium sulfate (final concentration, 50%). The precipitate obtained by centrifugation (10,000 × *g*, 10 min) was dissolved in 10 mM Tris·HCl, pH 8.1/0.2 mM DTT/1 mM EDTA and loaded on a DE52 cellulose column (2 cm^2 × 10 cm). The elution was carried out by the NaCl gradient (0–0.8 M), fractions between 0.3 and 0.4 M were pooled, and protein was precipitated by ammonium sulfate (final concentration, 50%). This step successfully removes a major portion of DNase activity and makes *in vitro* recombination assay feasible. Thus, we have tried to obtain the fraction that gives the highest recombination frequency in the assay. The precipitate was dissolved in a small amount of 10 mM Tris·HCl, pH 7.5/0.2 mM DTT/0.1 mM EDTA and dialyzed against the same buffer. The solution was loaded on a small single-strand DNA cellulose column (1.0 cm^2 × 2.0 cm). The bound protein was eluted with 0.5 M NaCl-containing buffer and concentrated with a Centricon after removal of NaCl by dialysis against 10 mM Tris·HCl (pH 7.6). The final concentration of protein was adjusted to 1.0 μg/μl.

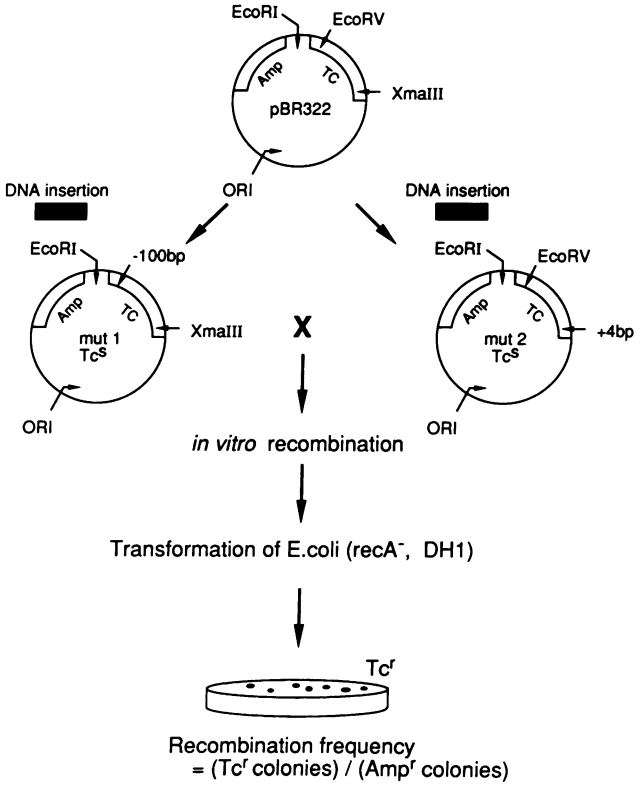

FIG. 1. *In vitro* recombination assay.

***In Vitro* Recombination Assay.** The reaction mixture for the *in vitro* recombination assay (21) contained the following in a total volume of 25 μl: 35 mM Hepes buffer (pH 7.8), 10 mM $MgCl_2$, 1 mM DTT, 2 mM spermidine, 5 mM ATP, 10% poly(vinyl alcohol), 10 μg of extract protein, and 1–2 μg of the mutated plasmids (mut 1 and mut 2). Incubation was carried out at 37°C for 60 min; DNA was then extracted with phenol/chloroform, precipitated with ethanol, and redissolved in 25 μl of H_2O. The mutated plasmids were used to transform *Escherichia coli* ($recA^-$, DH 1), and the ratio of tetracycline-resistant colonies over ampicillin-resistant colonies (Tc^r/Amp^r) was interpreted as recombination frequency (Fig. 1).

Filter Binding Assay. The nitrocellulose filter binding assay was employed to detect DNA–protein complexes. The ^{32}P-labeled DNA fragments were incubated with the cell extracts and passed through nitrocellulose under the condition that protein-bound DNAs bind to the filter but free DNA does not. The mixture (20 μl) contained 10 ng of ^{32}P-labeled deoxynucleotide triphosphate probe (specific activity was adjusted to 2×10^4 cpm/ng), 3 μg of poly(dI·dC), 2 μg of cold salmon sperm DNA (fragmented by a brief sonication to reduce the viscosity, resulting in ≈1000 bp), 4 μl of 5× concentrated buffer (100 mM Hepes, pH 7.6/0.5 mM EDTA/5 mM $MgCl_2$/200 mM KCl/2.5 mM DTT/20% Ficoll) and 5 μl of extract (5 μg of protein). Addition of 3 μg of poly(dI·dC) abolished the major nonspecific binding between protein and DNA. Further addition of poly(dI·dC) did not reduce the radioactivity on the filter. Two micrograms of salmon sperm DNA was sufficient for eliminating nonspecific binding. Addition of cold DNA fragments with homologous sequence reduced the radioactivity on the filter according to the dilution. The DNA–protein binding reaction was saturated within 10 min at 37°C, and a further 10 min of incubation did not change the radioactivity on the filter. The filter was washed with 2 ml of above buffer and dried. Radioactivity was assayed by a liquid scintillation counter. Nonspecific DNA–protein binding (the reaction with 2 μg of cold probe, about 100 times in quantity) was subtracted.

RESULTS

***In Vitro* Recombination.** A mut 1 or mut 2 alone did not produce any Tc^r colonies, nor did mut 1 and mut 2 when they were open-circularized or linearized. S1 nuclease treatment of plasmid DNAs after *in vitro* recombination (before transformation of *E. coli*) abolished activity, indicating that *in vitro* products contained a nick (ref. 21 and unpublished data). The background values were of the order of 10^{-6}. Production of Tc^r colonies from the two Tc^s plasmids, mut 1 and mut 2, depends upon the occurrence of recombination between two mutation sites in the tetracycline gene. Since the distance between those two mutation sites is constant (753 bp) (21), the recombination frequency (Tc^r/Amp^r) dependent on the crossing-over between two mutation sites should also be constant unless some other factors are interacting with inserted DNA sequences. In anti-paralleled orientation of the fragments, recombination frequency was reduced to <1 in 10.

As shown in Table 1, extracts from mouse spleen and bone marrow and from human HCC and melanoma stimulated the recombination frequency in the presence of either the 5.4-kb *Bam*HI or the 3.2-kb *Eco*RI fragment, compared to nonspecific control DNAs inserted at the same position in mut 1 and mut 2, including lily DNAs, M13 plasmid DNAs, or mouse ribosomal DNAs. The extracts from normal adult mouse (8 weeks) liver, muscle, and brain did not. Though absolute values of these increases are rather small, they were reproducible (three experiments always showed such an increase) and significant statistically ($P < 0.01$; *t* test). In this *in vitro* system, increasing the length of substrate DNA molecules did

Table 1. Recombination frequencies (Tc^r/Amp^r) stimulated by the insertion of HBV DNAs and control DNAs using cell extracts of various tissues

Source of cell extract	None*	Control DNA†		pBC4–5.4B HBV DNA‡	Wild-type HBV DNA§
		1.1 kb	5.1 kb		
Mouse					
Spleen	1.51 ± 0.07	1.63 ± 0.06	1.02 ± 0.06	3.10 ± 0.05¶	3.09 ± 0.09¶
Bone marrow	2.81 ± 0.28	1.57 ± 0.18	1.02 ± 0.21	5.54 ± 0.35¶	5.45 ± 0.13¶
Liver	0.04 ± 0.02	0.04 ± 0.02	0.08 ± 0.06	0.05 ± 0.02	0.06 ± 0.03
Muscle	0.07 ± 0.03	0.09 ± 0.05	0.06 ± 0.01	0.05 ± 0.04	0.06 ± 0.02
Brain	0.17 ± 0.03	0.18 ± 0.05	0.15 ± 0.07	0.21 ± 0.10	0.16 ± 0.05
Human					
HCC	3.23 ± 0.16	3.36 ± 0.04	1.01 ± 0.10	6.94 ± 0.79¶	6.68 ± 0.61¶
Melanoma	2.80 ± 0.39	2.57 ± 0.09	0.97 ± 0.20	5.13 ± 0.23¶	5.03 ± 0.13¶

Values are presented as (mean ± SD) × 10^{-4} from three experiments. Without cell extracts, by use of heat-treated extracts, or by use of mut 1 or mut 2 alone, recombination was rarely detected with the DH 1 strain of *E. coli* (the order of 10^{-6}). Mouse (Swiss random breed) tissues were obtained from 8-week-old animals and human HCC (HBV negative) and melanoma tissues were kindly provided by S. Yoshida. Note that increasing the length of the substrate DNA molecule decreases the frequency of recombination.

*Only mut 1 × mut 2 without insertion.

†Obtained from random clones of 1.1-kb and 5.1-kb lily DNAs (25).

‡5.4-kb *Bam*HI fragment of clone (22).

§3.2-kb *Eco*RI fragment of clone (24).

¶Significantly different from "None" and "Control DNA" at $P < 0.01$.

not, by itself, increase the frequency of recombination; rather, it produced decreases (see values of control fragment in Table 1 and unpublished data). Thus, the stimulation of recombination with insertion of HBV DNA fragments (5.4 kb and 3.2 kb; up to a 7-fold increase) appears to be sequence specific.

As a next step, we looked for the region in these fragments contributing to the stimulation of recombination. The 1076-bp (partial digestion), 440-bp, and 196-bp *Bgl* II fragments of pBC4-5.4B and 600-bp, 450-bp, and 300-bp *Bam*HI and/or *Bgl* II fragments of wild-type HBV were tested (Fig. 2). An increase in recombination frequency was observed with the 1076-bp and 196-bp fragments of pBC4-5.4B and a 600-bp fragment of wild-type HBV but not with the remaining fragments (Table 2). Note that the 196-bp fragment had 25-bp inverted repeats at both ends, as reported previously (16) (Fig. 2), but the 600-bp fragment did not, implying that the inverted repeat structure was not necessary to stimulate recombination in our system. Rather, specific sequences within the 196 bp fragment [spanning 2 bp from the 3′ end of the L-negative strand of HBV (1820) to downstream of pre-C (16), including DR1 (1824–1834), which is believed to be the origin of viral replication (26) and a preferred site in the viral genome for integration (16, 19, 27–29)] may be important.

DNA–Protein Binding. Treatment of cell extracts with proteases (10 μg of protease K per ml at 15°C, 5 min), exposure to heat (60°C, 5 min), or extreme pH (9.0–9.5) abolished recombination, suggesting involvement of specific cellular protein(s). Preliminary experiments using foot-print and gel-retardation methods showed the presence of a component(s) that specifically or preferably binds to these DNA fragments. After blocking potential nonspecific binding [presence of poly(dI·dC) at 3.0 μg per reaction mixture], significant binding was found between these three fragments (1076, 196, and 600 bp) and cell extracts from mouse spleen and bone marrow and from human HCC and melanoma (Table 2) but not to the control DNAs (lily DNAs and mouse ribosomal DNAs). These binding activities were inhibited by

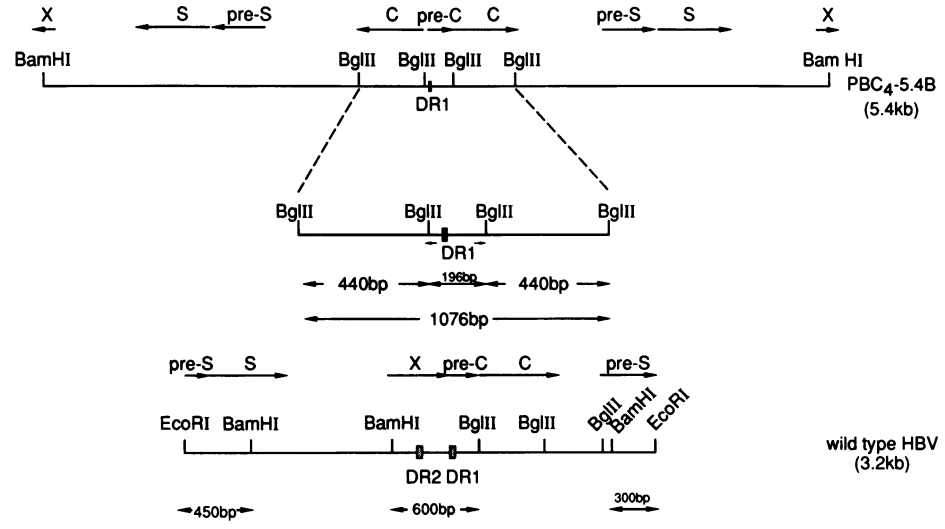

FIG. 2. HBV DNA fragments used in the *in vitro* recombination assay. (*Upper*) The 5.4-kb *Bam*HI, 1076-bp *Bgl* II (partial digestion), 440-bp *Bgl* II, and 196-bp *Bgl* II fragments were obtained from the pBC4–5.4B clone (22) [subtype adr (14)]. The 196-bp *Bgl* II fragment contains the DR1 and 25-bp inverted repeats at both ends (← →). (*Lower*) The 3.2-kb *Eco*RI, 600-bp *Bgl* II, 450-bp *Eco*RI–*Bam*HI, and 300-bp *Eco*RI–*Bam*HI fragments were obtained from wild-type HBV (subtype ayw_3) (24). S, surface gene; pre-S, pre-S gene; C, core gene; pre-C, pre-C gene; X, X gene. DR1 and DR2 indicate 11-bp directly repeated sequences 1 and 2, respectively.

Table 2. Effect of insertion of various HBV DNA fragments on *in vitro* recombination and DNA–protein binding assay

HBV DNA fragment, bp	Recombination frequency*	^{32}P bound to filter†
No insertion	1.56 ± 0.13	—
1076	4.60 ± 0.20‡	1610
440	1.31 ± 0.19	68
196	4.00 ± 0.32‡	317
600	4.70 ± 0.26‡	666
450	1.51 ± 0.21	39
300	1.50 ± 0.03	101

Experimental conditions are the same as in Table 1.
*Tc^r/Amp^r values are presented as (mean ± SD) × 10^{-4} using the same batch of mouse spleen extract. HBV DNA fragments were obtained as shown in Fig. 2.
†Radioactivity (cpm) bound to nitrocellulose filter.
‡Significantly different from "No insertion" at $P < 0.01$.

addition of the appropriate unlabeled DNA fragments (addition of 2×, 5×, and 10× the unlabeled DNA probes reduced the bound radioactivity by approximately 1/2, 1/5, and 1/10, respectively). Extracts from the cells exhibiting low *in vitro* recombination frequency did not show any significant binding to any of the fragments tested here (Table 2). These results suggest that stimulation of recombination by the insertion of a HBV DNA fragment involves a cellular protein(s) that binds to the specific fragment, though at present it is not known whether the binding protein(s) is the same as the recombinogenic protein(s).

DISCUSSION

A molecular mechanism that would explain the strong epidemiological link of persistent HBV infection with HCCs is still open to debate. There is also no reason why several different mechanisms cannot operate and contribute to hepatocarcinogenesis in different patients infected with the same virus. However, it is generally believed that toxicity-dependent degeneration–regeneration enhances initiation and promotion processes, not only during experimental (30) but also in human hepatocarcinogenesis (31, 32). Such a mechanism has also been shown recently in hepatocarcinogenesis of hereditary hepatitis in the rat (LEC rat) with abnormal copper accumulation (33) and even in transgenic mice with liver-specific expression of simian virus 40 large tumor antigen under the control of an albumin promoter (35), as shown in transgenic mice expressing the large S gene of HBV (6). The same mechanism may also be present in human hepatitis C virus-related hepatocarcinogenesis. If integrated HBV DNA promotes on-going genomic rearrangements in some hepatocellular lineages during chronic hepatitis, this could be an important synergistic factor leading to HBV-induced HCC. The experiments in this report provide evidence that HBV DNA, and specifically HBV DNA sequences containing the pre-C region spanning DR1, but no known HBV promoters or enhancers, stimulates homologous recombination in the presence of cellular protein extracts.

Previously we showed higher frequencies of *in vitro* recombination between circular DNA and linear DNA versus circular and circular and proposed that invasion of the linear molecule into a circular one was a initiating step in our system (21). Our preliminary studies also suggest that enzyme(s) activities in protein extract contain formation of a nick in twisted circular DNA, a D-loop produced by invasion of single-stranded DNA into double-stranded DNA, followed by the event of strand transfer, as considered in the first step of RecA-mediated recombination in prokaryotes and eukaryotes (34). It is also interesting to consider the breakage and reunion mechanism for recombination of virus–virus/virus–cell DNAs at the DR1 region, involving cellular enzymes, such as topoisomerase 1, as previously proposed (16) and recently demonstrated *in vitro* (29).

In conclusion, we propose that HBV can cause an increase in the incidence of liver cancer by a combination of two mechanisms: (*i*) cell killing and stimulation of mitosis that can accumulate the number of events necessary for transformation and (*ii*) increase of chromosomal instability by the insertion of HBV DNAs, apparently mediated by proteins that can stimulate recombination during chronic hepatitis. Our present results paradoxically lead to the hypothesis that integrated HBV DNA might be stable in host DNA without a putative recombinogenic region of HBV DNA or recombinogenic cellular protein(s)—for example, in the healthy HBV carrier state. This mechanism seems to explain why among HBV carriers, HCCs usually develop only in patients with chronic hepatitis and/or cirrhosis. Finally, determination of the putative recombinogenic HBV DNA sequence(s) and isolation of HBV DNA-binding cellular protein(s), involved in recombination, which are abundant in dividing cells but not in resting cells, provide an exciting approach to understanding the genomic instability of cancer cells. Our *in vitro* recombination system may be a useful tool for this research.

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1. Beasley, R. P. (1982) *Hepatology* **2**, 21S–26S.
2. Brand, N., Petkovich, M., Krust, A., Chambon, P., DeThe, H., Marchio, A., Tiollais, P. & Dejean, A. (1988) *Nature (London)* **332**, 850–853.
3. Wang, J., Chenivesse, X., Henglein, B. & Bréchot, C. (1990) *Nature (London)* **343**, 555–557.
4. Tiollais, P. & Buendia, M.-A. (1991) *Sci. Am.* **264** (4), 116–123.
5. Kim, C.-M., Koike, K., Saito, I., Miyamura, T. & Jay, G. (1991) *Nature (London)* **351**, 317–320.
6. Chisari, F. V., Fillippi, P., Buras, J., McLachlan, A., Popper, H., Pinkert, R. D., Palmiter, R. D. & Brinster, R. L. (1989) *Cell* **59**, 1145–1156.
7. Wang, H. P. & Rogler, C. E. (1988) *Cell Genet.* **48**, 72–78.
8. Buetow, K. H., Murray, J. C., Israel, J. L., London, W. T., Smith, M., Kew, M., Blanquet, V., Brechot, C., Redeker, A. & Gonvindarajah, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8852–8856.
9. Tsuda, H., Zhang, W., Shimosato, Y., Yokota, J., Terada, M., Sugimura, T., Miyamura, T. & Hirohashi, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6791–6794.
10. Slagel, B. L., Zhou, Y.-Z. & Butel, J. S. (1991) *Cancer Res.* **51**, 49–54.
11. Fujimori, M., Tokino, T., Hino, O., Kitagawa, T., Imamura, T., Okamoto, E., Mitsunobu, M., Ishikawa, T., Nakagawa, H., Harada, H., Yagura, M., Matsubara, K. & Nakamura, Y. (1991) *Cancer Res.* **51**, 89–93.
12. Urano, Y., Watanabe, K., Lin, C. C., Hino, O. & Tamaoki, T. (1991) *Cancer Res.* **51**, 1460–1464.
13. Knudson, A. G. (1986) *Annu. Rev. Genet.* **20**, 231–251.
14. Hino, O., Shows, T. B. & Rogler, C. E. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8338–8342.
15. Henderson, A. S., Ripley, S., Hino, O. & Rogler, C. E. (1988) *Cancer Genet. Cytogenet.* **30**, 269–275.
16. Hino, O., Ohtake, K. & Rogler, C. E. (1989) *J. Virol.* **63**, 2638–2643.
17. Rogler, C. E., Sherman, M., Su, C. Y., Shafritz, D. A., Summers, J., Shows, T. B., Henderson, A. & Kew, M. (1985) *Science* **230**, 319–322.
18. Mizusawa, H., Taira, M., Yaginuma, K., Kobayashi, M., Yoshida, E. & Koike, K. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 208–212.
19. Nagaya, T., Nakamura, T., Tokino, T., Tsurimoto, T., Imai,

M., Mayumi, T., Kamino, K., Yamamura, K. & Matsubara, K. (1987) *Genes Dev.* **1,** 773–782.
20. Tokino, T., Fukushige, S., Nakamura, T., Nagaya, T., Murotsu, T., Shiga, J., Aoki, N. & Matsubara, K. (1987) *J. Virol.* **61,** 3848–3854.
21. Hotta, Y., Tabata, S., Bouchard, R. A., Pinon, R. & Stern, H. (1985) *Chromosoma* **15,** 140–151.
22. Hino, O., Nomura, K., Ohtake, K., Kitagawa, T., Sugano, H., Kimura, S., Yokoyama, M. & Katsuki, M. (1986) *Proc. Jpn. Acad. Sci. B* **62,** 355–358.
23. Hino, O., Nomura, K., Ohtake, K., Kawaguchi, T., Sugano, H. & Kitagawa, T. (1989) *Cancer Genet. Cytogenet.* **37,** 273–278.
24. Pasek, M., Goto, T., Gilbert, W., Zink, B., Schaller, H., Mackay, P., Leadbetter, G. & Murray, K. (1979) *Nature (London)* **282,** 575–579.
25. Hotta, Y., Tabata, S., Stubbs, L. & Stern, H. (1985) *Cell* **40,** 785–793.
26. Seegar, C., Ganem, D. & Varmus, H. (1986) *Science* **232,** 477–484.
27. Dejean, A., Sonigo, P., Wain-Hobson, S. & Tiollais, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5350–5354.
28. Shin, C., Burke, K., Chow, M.-J., Zeldis, J. B., Yang, C.-S., Lee, C.-S., Isselbacher, K. J., Wands, J. R. & Goodman, H. M. (1987) *J. Virol.* **61,** 3491–3498.
29. Wang, H.-P. & Rogler, C. E. (1991) *J. Virol.* **65,** 2381–2392.
30. Moore, M. A. & Kitagawa, T. (1986) *Int. Rev. Cytol.* **101,** 125–173.
31. London, W. T. & Blumberg, B. S. (1982) *Hepatology* **2,** 10S–14S.
32. Preston-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A. & Henderson, B. E. (1990) *Cancer Res.* **50,** 7415–7421.
33. Li, Y., Togashi, Y., Sato, S., Emoto, T., Kang, J.-H., Takeichi, N., Kobayashi, H., Kojima, Y., Une, Y. & Uchino, J. (1991) *J. Clin. Invest.* **87,** 1858–1861.
34. Higashitani, A., Tabata, S., Ogawa, T., Ogawa, H., Shibata, M. & Hotta, Y. (1990) *Exp. Cell Res.* **186,** 317–323.
35. Hino, O., Kitagawa, T., Nomura, K., Ohtake, K., Cui, L., Furuta, Y. & Aizawa, S. (1991) *Jpn. J. Cancer Res.*, in press.