JOURNAL OF VIROLOGY, Feb. 1988, p. 629–632
0022-538X/88/020629-04$02.00/0

Vol. 62, No. 2

Rearrangement of a Common Cellular DNA Domain on Chromosome 4 in Human Primary Liver Tumors

C. PASQUINELLI,[1,2] F. GARREAU,[1,2] L. BOUGUELERET,[3] E. CARIANI,[2] K. H. GRZESCHIK,[4] V. THIERS,[1] O. CROISSANT,[5] M. HADCHOUEL,[1] P. TIOLLAIS,[1] AND C. BRÉCHOT[1,2]*

Unité de Recombinaison et Expression Génétique,[1] Unité Informatique Scientifique,[3] and Unité des Papillomes, Institut Pasteur Paris,[5] and Unité de Biochimie Pharmaco-Métabolique, CHU Necker,[2] Paris 75015, France, and Institut für Humangenetik, 4400 Münster, Federal Republic of Germany[4]

Received 13 July 1987/Accepted 25 October 1987

Hepatitis B virus (HBV) DNA integration has been shown to occur frequently in human hepatocellular carcinomas. We have investigated whether common cellular DNA domains might be rearranged, possibly by HBV integration, in human primary liver tumors. Unique cellular DNA sequences adjacent to an HBV integration site were isolated from a patient with hepatitis B surface antigen-positive hepatocellular carcinoma. These probes detected rearrangement of this cellular region of chromosomal DNA in 3 of 50 additional primary liver tumors studied. Of these three tumor samples, two contained HBV DNA, without an apparent link between the viral DNA and the rearranged allele; HBV DNA sequences were not detected in the third tumor sample. By use of a panel of somatic cell hybrids, these unique cellular DNA sequences were shown to be located on chromosome 4. Therefore, this region of chromosomal DNA might be implicated in the formation of different tumors at one step of liver cell transformation, possibly related to HBV integration.

Extensive epidemiological studies clearly have indicated the association between hepatitis B virus (HBV) chronic infection and the development of hepatocellular carcinoma (HCC) (1). In addition, the viral DNA sequences were shown to be present, integrated in the host genome, in the tumor cells of patients with HCC (2, 6–9, 12, 13, 15–20). By cloning HBV and adjacent cellular DNA sequences, it is possible to investigate whether some regions of chromosomal DNA are rearranged in different tumors.

We first analyzed tissue samples obtained from patient A, a chronic carrier of HBV with HCC. Southern blot analysis of chromosomal DNA extracted from the tumor (Fig. 1A) showed the presence of at least four integrated HBV genomes (lanes E and F). Integrated HBV DNA was also identified in the peritumorous liver tissue (lanes C and D) but not in the nontumorous liver tissue (lanes A and B). HBV DNA replication was evident only in the nontumorous tissues (Fig. 1A, panels 1 and 2). *Eco*RI digests of chromosomal DNA from both tumorous and peritumorous liver tissue showed, after hybridization with the HBV probe, two DNA fragments of 7 and 8 kilobases (kb) containing HBV and cellular DNA sequences (Fig. 1A, lanes D to F). From a genomic library of tumor DNA of patient A, three independent clones containing HBV DNA along with flanking cellular DNA sequences were obtained. One of these clones (λ TDI6, Fig. 1B), including the 8-kb *Eco*RI DNA fragment, was further analyzed. HBV DNA sequences (2.6 kb) extended from the pre-S1 region to the cohesive ends of the viral genome and lacked the core gene sequence. Comparison of the restriction maps of the integrated and cloned HBV, as well as heteroduplex analysis (data not shown), did not reveal apparent deletions or rearrangements in the viral DNA. Because of a rearrangement, which occurred during the cloning procedure, at the right side of the λ TDI6 insert, it was not possible to determine with certainty the left junction DNA sequence. A 0.4-kb cellular DNA fragment of λ TDI6 insert free of repeat sequences was subcloned in the

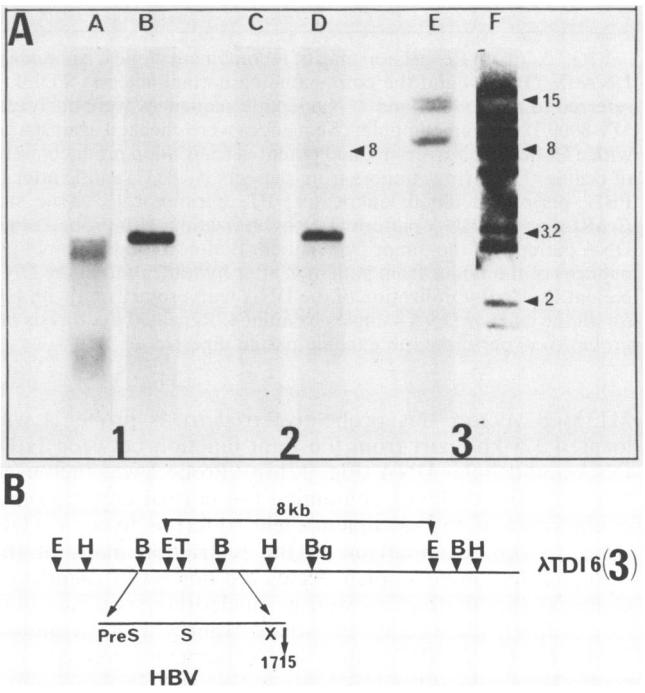

FIG. 1. (A) Southern blot analysis of cellular DNA from the liver of patient A after hybridization of the DNA with HBV probe. Chromosomal DNA was extracted as previously described (2) and analyzed with the Southern blot procedure. The numbers 1, 2, and 3 correspond to the nontumorous, peritumorous, and tumorous liver tissue samples, respectively. Lanes: A, C, and E, *Hin*dIII digestion; B, D, and F, *Eco*RI digestion. The arrowheads indicate the number of kilobases. (B) Restriction map of a recombinant clone (λ TDI6) isolated after hybridization with HBV from panel 3 [λ TDI6(3)] tumor samples containing integrated HBV DNA. The genomic library was generated after partial digestion with *Sau*3A and ligation to *Bam*HI-digested λ L47-1 (7). The nucleotide sequence was obtained by the dideoxy chain termination procedure (6).

* Corresponding author.

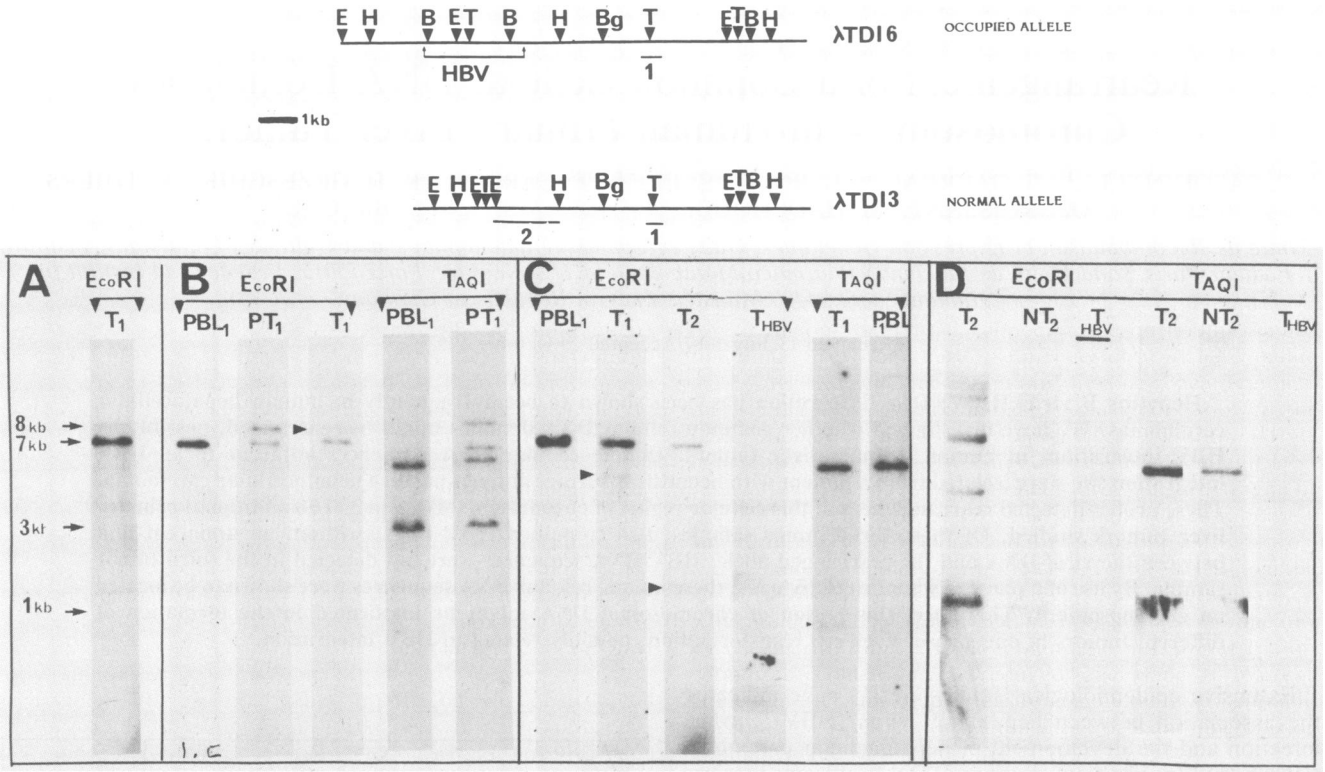

FIG. 2. (Top) Restriction map of recombinant clones, isolated from the tumor from patient A (Fig. 1A, panel 3), containing integrated HBV DNA [λ TDI6(3)] and the corresponding normal allele (λ TDI3). Cellular DNA sequences free of repeat sequences were isolated and are referred to as probes 1 and 2. Nucleotide sequences were analyzed with the 5 SASIP programming package (3, 5). Programs were run on an MV 8000 32-bit minicomputer. Sequences were checked against a gene bank (DNA sequences were processed in fragments 100 base pairs long with a 50-base-pair overlap), and potential translation products were checked against NBRF and PG-trans (4). (Bottom) Southern blot analysis of cellular DNA from tumors from patients A, B, C, and D after hybridization of DNA with probes 1 and 2 and with HBV. Abbreviations: PBL, peripheral blood leukocytes; PT, peritumorous tissue samples; T, tumorous tissue. (A) Tumor from patient A. Shown is the *Eco*RI-digested-DNA pattern after hybridization with probe 1; the same DNA fragment hybridized with probes 1 and 2 and with HBV. (B) DNA patterns of the tumor from patient B after hybridization of the DNA with probe 1; HBV DNA was not detected in the tumor. (C) DNA patterns of the tumor from patient C after hybridization of the DNA with probes 1 and 2 and with HBV. (D) DNA pattern of the tumor from patient D after hybridization of the DNA with probe 2 and with HBV. The absence of contaminating bacterial DNA sequences was checked for all the cellular DNA samples examined. Repeated hydrolysis of the cellular DNA, as well as hybridization with internal controls (albumin probe), was performed to exclude partial digests.

M13Mp8 vector; this probe (referred to as probe 1) was located 3.5 kb apart from the right junction between HBV DNA and cellular DNA (Fig. 2, top). Probe 1 was then used to obtain three clones containing the normal chromosomal counterpart of the occupied allele, referred to as λ TDI3 (Fig. 2, top). Comparison of the restriction maps of the occupied and nonoccupied alleles did not reveal significant changes; this finding, as well as nucleotide sequence data (see below), allowed us to isolate from λ TDI3 a unique 1.9-kb *Eco*RI-*Hin*dIII cellular DNA fragment (referred to as probe 2) located at the HBV integration site. Probes 1 and 2 showed, upon Southern blotting of normal cellular DNA, a 7-kb DNA fragment in the *Eco*RI digest (Fig. 2, bottom); this fragment was included in the λ TDI3 recombinant. *Taq*I digestion yielded a restriction fragment length polymorphism; probe 1 showed a 5-kb DNA fragment, a 2.5-kb fragment, or both (this was the case in tumor DNA from patient A). Probe 2 showed a 5-kb DNA fragment (as in tumor DNA from patient A), a 2.3-kb fragment, or both.

To determine whether this region of cellular DNA might be rearranged in different primary liver tumors, DNAs from 40 HCCs and from 10 benign liver tumors were digested with *Eco*RI and analyzed by the Southern blot technique for sequences hybridizing with probes 1 and 2. As a control, 40 normal DNAs were also tested. All 50 tumor DNAs, as well as the 40 DNA controls, revealed the expected 7-kb *Eco*RI fragment with both probes 1 and 2. Four tumors (including the tumor from patient A) revealed additional DNA fragments at different positions (Fig. 2, bottom); these tumors, included three HCCs (patients A, B, and C) and one adenoma (10) (patient D) containing some areas of highly dysplastic liver cells. DNAs from the peripheral leukocytes or nontumorous liver tissue were available in the four subjects and showed only the 7-kb *Eco*RI fragment; thus, the results could not be explained by a polymorphism but rather reflected rearrangement of this cellular DNA region in the tumor cells. For the tumor from patient B, the *Eco*RI-digested-DNA pattern showed with both probes 1 and 2 an additional 8-kb DNA fragment (Fig. 2B). *Taq*I digestion yielded two additional bands (7- and 9-kb DNA fragments; Fig. 2B). For the tumor from patient C, probe 1 revealed additional 5-kb *Eco*RI and 1.2-kb *Taq*I DNA fragments; probe 2 revealed a 2.2-kb fragment in the *Eco*RI-digested-DNA pattern and did not reveal an additional band in the *Taq*I-digested-DNA pattern (Fig. 2C). These results suggested that, in this case, rearrangement occurred in the 7-kb

TABLE 1. Chromosomal localization of the cellular probes (probes 1 and 2) on chromosome 4 by using somatic cell hybrids

Clone	Presence of the following chromosome (+) or of a chromosome fragment or marker (o):																								Chromosome marker	Test result
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y		
RAG GM610 5-23	+		+	+	+	o	+	+			+		+		+	+		o	+	+	+		+		18p+	+
RAG Ru la-5-11			+	+			o							+							+	+			7q	+
RAG MH 8-7	+		+	+	o		o	+				+	+	+	o			+					+		15q−	+
A9 5U 1-2		+	o			+							+	+			o	+			+				3p+	−
RAG GM 194 6-13			o	+			o					+	+	+	o								o +	+	7q−	+
RAG GM 194 5-5			o	+	+	+	+			+		+	+	+		+				+	+	+		o		−
RAG 1 AnLy	+		+	+	+	+	+		o			+						+			+		o		9pt	+
RAG RU 4-13	o		+		o	+	o			o				o			+		o		+	+	o	+	7p	−
A9 GM 89 9c-7			+	+			+				+				+	(+)[a]		+		o	+	+	o		Xq−	+
RAG GM 97 8-13	o			+					+					+			+					+	o		1q−	+
A9 Call 1-13		o				o							+	+	+			+			+				6p+	−
A9 JT 2-21-14					+	o	o					+			+	+	+	+			+		+		7p+	−
RAG GN 6a-8-4						+	o														o					+
A9 JT 2-18					+	o	+										+	+			+				6q−	−
5387 3 cl.10							+																			−
A9 Call 1-9-9		o				o							+	+	+			+	+	+	+	+			2p−	−
GM 3104											Human			Control												+
A9											Mouse			Control												−

[a] (+), Chromosome present in more than 30%, but not more than 50%, of the cells.

*Eco*RI fragment, close to the *Hin*dIII site. For the tumor from patient D, a complex DNA pattern was observed (Fig. 2D); *Eco*RI digestion revealed several intense additional bands (17, 12, 5, and 2.5 kb) after hybridization with probe 2 but not with probe 1. However, additional fragments were not identified in the *Taq*I-digested-DNA pattern with both probes 1 and 2.

Hepatitis B surface antigen was detected in the serum of two subjects (patients A and D), whereas the other two (patients B and C) had no HBV serological markers. HBV DNA sequences were not identified in the tumor from patient B. For the tumor from patient A, an 8-kb DNA fragment was shown in the *Eco*RI-digested-DNA pattern with HBV and with probes 1 and 2. For the tumors from patients C and D, the DNA fragments generated by different restriction enzymes and identified with HBV and probes 1 and 2 were of different sizes; therefore, there was apparently no link between the viral DNA and the rearranged allele in these samples. HBV DNA sequences were not detected in the tumor from patient B.

In the tumors from patients A and C (Fig. 2A and C), the intensities of hybridization of probes 1 and 2 to the additional DNA fragments were low compared with the normal 7-kb *Eco*RI DNA fragment. Thus, rearrangement in this region of chromosomal DNA occurred in a limited percentage of the tumor cells. In the DNA patterns of tumors from patients B and D, most of the cells appeared to contain a rearrangement in this region (as evident in the peritumorous [patient B] and tumorous [patient D] liver samples).

The chromosomal localization of probes 1 and 2 was then studied by using human-mouse and human-Chinese hamster hybrid cells (a gift from K. H. Grzeschick and A. Dejean); the results are consistent with the localization of the DNA sequences on chromosome 4 (Table 1). The nucleotide sequence of a 2.3-kb *Hin*dIII DNA fragment of the normal allele (λ TDI3), including the integration site, was determined (Fig. 2, top); in addition, the sequence of an *Eco*RI-*Hin*dIII fragment of the occupied allele (λ TDI6; Fig. 2, top), including the right junction between HBV and cellular DNA, was also determined. The nucleotide sequence of the normal allele revealed the presence of short open reading frames (up to 300 base pairs) but no evidence for their being exonlike sequences (4). There was no significant homology with any cellular gene. The frequency of GC nucleotides was not significantly increased (21). The nucleotide sequence 1.1 kb from the HBV integration site showed a GTGGAAT sequence repeated with an interval of eight nucleotides (GTGGAATCAATAACAGTGGAATC); this sequence had a strong homology to the GTGG$^{AAA}_{TTT}$G core-enhancer consensus sequence (11, 14). In the occupied allele, the virus-host junction mapped at position 1715 on the viral genome, in the cohesive-ends region (8). Comparison of the cellular DNA sequences in the occupied and normal alleles did not reveal significant changes.

This study facilitated the identification of rearrangements of a common cellular DNA domain in the human primary liver tumors; rearrangement was detected in 4 of the 50 tumor samples tested. Therefore, this region of chromosomal DNA, located on chromosome 4, might be involved in one of the steps in cell transformation. This DNA domain does not seem to be frequently involved since its rearrangement was identified in only 10% of the tumors studied; however, this uninvolvement might be reassessed with probes covering a larger DNA region. In view of this, it is also noteworthy that in some cases this rearrangement was detected in a low percentage of cells and in liver tissue samples adjacent to the tumors. This finding might be interpreted as evidence for the involvement of cellular DNA rearrangement at an early step in liver cell transformation; alternatively, this rearrangement might occur in malignant cells, either being disseminated from the bulk of the tumor or arising in a multifocal tumor. Preliminary studies, using the two cellular probes described here, did not show transcription of these DNA sequences in normal or tumorous livers (data not shown).

Although HBV DNA was present in three of the four liver tumors that had DNA rearrangements, in only one tumor was the viral DNA clearly linked to the modified allele. Thus, it appears that common molecular events might be related to viral as well as nonviral liver carcinogenesis.

We thank Anne Dejean and Marie-Annick Buendia for help and criticisms during this study. We are also grateful to Tarik Möröy for helpful discussions and to Ana Cova for typing the manuscript.

Work supported by grants from the Association pour la recherche sur le cancer (no. 6252) and Ligne Nationale contre le cancer.

LITERATURE CITED

1. **Beasley, R. P., and L. Y. Hwang.** 1984. Epidemiology of hepatocellular carcinoma, p. 209–214. *In* G. N. Vyas, J. Dienstag, and J. Hoofnagle (ed.), Viral hepatitis and liver disease. Grune & Stratton, New York.
2. **Bréchot, C., C. Pourcel, A. Louise, B. Rain, and P. Tiollais.** 1980. Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma. Nature (London) **286**:533–535.
3. **Claverie, J. M.** 1984. A common philosophy and Fortran 77 software package for implementing and searching sequence data bases. Nucleic Acids Res. **12**:397.
4. **Claverie, J. M., and L. Bougueleret.** 1986. Heuristic informational analysis of sequences. Nucleic Acids Res. **14**:179–195.
5. **Claverie, J. M., and I. Sauvaget.** 1985. A new protein data bank. Nature (London) **318**:19.
6. **Dejean, A., L. Bougueleret, K. H. Grzeschick, and P. Tiollais.** 1986. Hepatitis B virus DNA integration in a sequence homologous to v-erb-A and steroid receptor genes in a hepatocellular carcinoma. Nature (London) **322**:70–72.
7. **Dejean, A., C. Bréchot, P. Tiollais, and S. Wain-Hobson.** 1983. Characterization of integrated hepatitis B viral DNA cloned from a human hepatoma and the hepatoma-derived cell line PLC/PRF/5. Proc. Natl. Acad. Sci. USA **80**:2505–2509.
8. **Dejean, A., P. Sonigo, S. Wain-Hobson, and P. Tiollais.** 1984. Specific hepatitis B virus integration in hepatocellular carcinoma DNA through a viral 11 base pair direct repeat. Proc. Natl. Acad. Sci. USA **81**:5350–5354.
9. **Edman, J. C., P. Gray, P. Valenzuela, L. B. Rall, and W. J. Rutter.** 1980. Integration of hepatitis B virus sequences and their expression in a human hepatoma cell. Nature (London) **286**:535–538.
10. **Edmonson, H. A., and R. L. Peters.** 1982. Neoplasms of the liver. *In* L. Schiff and E. Schiff (ed.), Diseases of the liver. Lippincott Company, Philadelphia.
11. **Gillies, S. D., S. L. Morrison, V. T. Oi, and S. Tonegawa.** 1983. A tissue specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell **33**:717–728.
12. **Hino, O., T. B. Shows, and C. E. Rogler.** 1986. Hepatitis B virus integration site in hepatocellular carcinoma at chromosome 17–18 translocation. Proc. Natl. Acad. Sci. USA **83**:8338–8342.
13. **Koshy, R., S. Koch, A. Freytag von Loringhoven, R. Kahmann, K. Murray, and P. H. Hofschneider.** 1983. Integration of hepatitis B virus DNA: evidence for integration in the single-stranded gap. Cell **34**:215–223.
14. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by the repeats from simian virus 40 and moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
15. **Marion, P. L., F. H. Salazar, J. J. Alexander, and W. S. Robinson.** 1980. State of hepatitis B viral DNA in a human hepatoma cell line. J. Virol. **33**:795–806.
16. **Möröy, T., A. Marchio, J. Etiemble, C. Trépo, P. Tiollais, and M. A. Buendia.** 1986. Rearrangement and enhanced expression of c-myc in hepatocellular carcinoma of hepatitis virus infected woodchucks. Nature (London) **324**:276–279.
17. **Rogler, C. E., M. Sherman, C. Y. Su, D. A. Shafritz, J. Summers, T. B. Shows, A. Henderson, and M. Kew.** 1985. Deletion in chromosome 11 p associated with a hepatitis B integration site in hepatocellular carcinoma. Science **239**: 319–322.
18. **Shafritz, D. A., D. Shouval, H. I. Cherman, S. J. Hadziyannis, and M. C. Kew.** 1981. Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. Studies in percutaneous liver biopsies and post-mortem tissue specimens. New Engl. J. Med. **305**: 1067–1073.
19. **Shaul, Y., P. D. Garcia, S. Schonberg, and W. J. Rutter.** 1986. Integration of hepatitis B virus in chromosome-specific satellite sequences. J. Virol. **59**:731–734.
20. **Yaginuma, K., M. Kobayashi, E. Yoshida, and K. Koike.** 1985. Hepatitis B virus integration in hepatocellular carcinoma DNA: duplication of cellular flanking sequences at the integration site. Proc. Natl. Acad. Sci. USA **82**:4458–4462.
21. **Zerial, M., J. Salinas, J. Filip, and J. Bernardi.** 1986. Genomic localization of hepatitis B virus in a human hepatoma cell line. Nucleic Acids Res. **14**:8373.