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Structural Rearrangement of Integrated Hepatitis B Virus DNA as well as Cellular Flanking DNA Is Present in Chronically Infected Hepatic Tissues

SHINAKO TAKADA,[1] YASUHIRO GOTOH,[1,2] SHIGEKI HAYASHI,[3] MICHIHIRO YOSHIDA,[4] AND KATSURO KOIKE[1]*

Department of Gene Research, Cancer Institute, Kami-Ikebukuro Toshima-ku, Tokyo 170,[1] Department of Pediatrics, Nagoya University School of Medicine, Shouwa-ku, Nagoya 466,[2] First Department of Internal Medicine, Tokyo University School of Medicine, Hongo, Bunkyo-ku, Tokyo 113,[3] and Faculty of Science, Hokkaido University, Kita-ku, Sapporo 060,[4] Japan

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Cellular DNAs from human livers chronically infected with hepatitis B virus (HBV) were analyzed by Southern blot hybridization for the presence of integrated HBV DNA. In 15 of 16 chronically infected hepatic tissues, random HBV DNA integration was evident. By molecular cloning and structural analyses of 19 integrants from three chronically infected hepatic tissues, deletion of cellular flanking DNA in all cases and rearrangement of HBV DNA with inverted duplication or translocation of cellular flanking DNA at the virus-cell junction in some cases were noted. Thus, the rearrangement of HBV DNA or cellular flanking DNA is not a specific incident of hepatocellular carcinoma formation. Detailed analyses of various integrants bearing rearranged viral DNA failed to indicate any gross structural alteration in cellular DNA, except for a small deletion at the integration site, indicating that viral DNA rearrangement with inverted duplication possibly occurs before integration of HBV DNA. Based on nucleotide sequencing analyses of virus-virus junctions, a one- to three-nucleotide identity was found. A mechanism for this inverted duplication of HBV DNA is proposed in which illegitimate recombination between two complementary viral strands may take place by means of a nucleotide identity at the junction site in a weakly homologous region (patchy homology) on one side of adjoining viral sequences. For virus-cell junctions, the mechanism may be basically similar to that for virus-virus junctions.

Hepatitis B virus (HBV) is a causative agent for acute and chronic hepatitis in humans, and its chronic infection is closely related to the development of hepatocellular carcinoma (HCC) (30). The integration of HBV DNA occurs in HCC tissues at high frequency (11, 31) and is considered to be importantly involved in the initial stage of hepatocarcinogenesis. Structural analyses of integrated HBV DNAs were done on many HCC samples (5, 9, 12, 15, 16, 20, 32–35), and several characteristics became evident. The cellular site of HBV integration is random at both the cytogenetic and DNA sequence levels. One end of the integrated HBV DNA is close to the 5′-end region of the negative or positive viral strand (DR1 or DR2, respectively). Various integrated structures could be seen, such as the following: (i) a colinear structure of HBV DNA having the 5′-end region of the negative or positive viral strand as one end with or without cellular DNA rearrangement; (ii) an inversely duplicated structure of HBV DNA together with cellular flanking DNA; and (iii) a highly rearranged structure of HBV DNA without rearrangement of cellular flanking DNAs. However, no common structure has been found in HCC at high frequency so far.

Chronic hepatitis is considered to be a premalignant stage of HCC, since HCC frequently developes via chronic hepatitis and carriers experimentaly infected by woodchuck hepatitis virus (WHV) developed HCC in all cases (18). Southern blot analyses by several investigators (2, 3, 25) have demonstrated HBV DNA integration even in some chronically and acutely infected hepatic tissues. Thus, the structural features of HBV DNA integration in chronically infected hepatic tissues should be examined in detail and compared with those of HCC for clarification of the causal relationship between HBV integration and the development of HCC.

In previous studies on samples chronically infected with HBV for integration by molecular cloning, only two integrants from a human chronic active hepatitis (33) and one from a woodchuck hepatocyte chronically infected by WHV (22) were examined. The data indicated a colinear structure of viral DNA having the 5′-end region of the negative strand as one end of integrated viral DNA. Whether there is a structural difference between integrated viral DNAs from chronic hepatitis and HCC is a point yet to be resolved. In chronically infected woodchuck or ground squirrel liver, there were found extrachromosomal circular DNAs of more than two genome equivalents with extensive rearrangement, the so-called novel form of WHV or ground squirrel hepatitis virus (13, 21). This novel form may be integrated to cellular DNA at least under certain conditions.

In the present study, chronically infected liver samples were examined in detail by Southern blot analysis and molecular cloning to ascertain the structural features of integrated HBV DNAs. HBV DNA integration in most tissue samples and rearrangement of viral DNA and/or cellular flanking DNA were found. The rearrangement of HBV DNA as well as cellular flanking DNA appears not to be specific for HCC cells. Moreover, some data were obtained indicating that viral DNA rearrangement possibly occurs before integration. A possible mechanism for viral DNA rearrangement and integration is discussed.

* Corresponding author.

TABLE 1. Histological, serological, and hybridization results

Patient	Sex	Age (yrs)	Histological diagnosis[a]	Serological marker(s)[b]	HBV DNA in liver	
					Integrated	Free
N1	M	6	CAH	sAg/cAb/eAg	+	+
N2	M	5	CAH	sAg/cAb/eAg	+	+
NG	F	12	AC	sAg/eAg	+	+
NO	M	15	CAH	eAg	+	+
NS	M	15	CH	sAg/eAb	+	+
T1	M	23	CAH	sAg/eAg	+	+
T2	M	42	CAH	sAg/eAg	+	+
T3	M	39	CAH	sAg/cAb/eAg	?	+
T4	M	44	CH	sAg/cAb/eAg	+	+
T5	M	28	CAH	sAg/eAg	+	+
T6	M	36	CH	sAg/cAb/eAg	+	+
Sa	F	20	CH	sAg/eAg	+	+
Mo	M		CH	cAb/eAg	+	+
Ta	M	27	CAH, LC		+	+
Se	M	41	CH	sAg/eAg	+	+
Ni	M	49	CH	sAg/eAg	+	+

[a] CAH, Chronic active hepatitis; AC, asymptomatic carrier; CH, chronic hepatitis; LC, liver cirrhosis.

[b] sAg, HBV surface antigen; cAb, antibody to HBV core antigen; eAg, HBV e antigen.

MATERIALS AND METHODS

Tissue samples. Tissue samples were obtained surgically or by needle biopsy and stored in liquid nitrogen or a deep freezer (−80°C) until DNA extraction. The samples are listed in Table 1. No patient had a tumor at the time of examination.

Blot hybridization. Blot hybridization was performed by the method of Southern (28). The ^{32}P-labeled hybridization probe of HBV DNA or cellular flanking DNA was made by nick translation (19).

Cloning and sequencing of integrated HBV DNA. Cellular DNA was extracted from chronically infected hepatic samples as previously described (10). N2 DNA and T4 DNA were completely digested with *Hin*dIII and 6- to 23-kilobase (kb) (N2) or 4.4- to 23-kb (T4) fragments were fractionated by agarose gel electrophoresis and then ligated to the cloning vector Charon21AM, a modified Charon21A for cloning *Hin*dIII fragments (34). N1 DNA was partially digested with *Sau*3A, and 6.8- to 23-kb fragments were ligated to the vector EMBL3. After in vitro packaging, recombinant libraries were screened with ^{32}P-labeled HBV DNA by the procedure of Benton and Davis (1) and positive plaques were selected for the following experiments.

Appropriate restriction fragments containing virus-virus or virus-cell junctions were isolated from each clone. Some of them were directly subjected to sequencing analyses by the chemical modification method (14), and the others were subcloned to plasmid pUC19 for sequence analysis by the chain termination method (23).

Cloning of cellular counterpart DNAs. Cellular counterpart DNAs were isolated by screening the gene library constructed with a Charon21AM vector and a 2- to 3-kb *Hin*dIII fragment of normal human thymus DNA (for N2-7) or with an EMBL3 vector and *Sau*3A partially digested fragments from the HBV-negative hepatoma cell line HuH-7 (for HCY-23).

RESULTS

Southern blot analyses. Cellular DNAs from chronically infected hepatic tissues of patients of different ages were analyzed by Southern blot hybridization with HBV DNA as the probe. The data for these samples are summarized in Table 1, and some blot hybridization results are shown in Fig. 1. All the samples except T3 (Fig. 1, lane 9) exhibited hybridization signals in the high-molecular-weight region when blot hybridization was conducted without restriction enzyme digestion. When DNAs were digested with *Hin*dIII, which does not cut the inside of HBV DNA, the hybridization signals became dispersed. No discrete band could be found for any sample analyzed, indicating that most of the chronically infected hepatic tissues have HBV DNA integrated to cellular DNA and that heterogeneous cell populations exist with respect to the HBV DNA integration site. Hybridization signals in the low-molecular-weight region showed free viral DNA released from virus particles by proteinase K digestion. Since viral DNA integration was noted even in a sample from an asymptomatic carrier (NG in

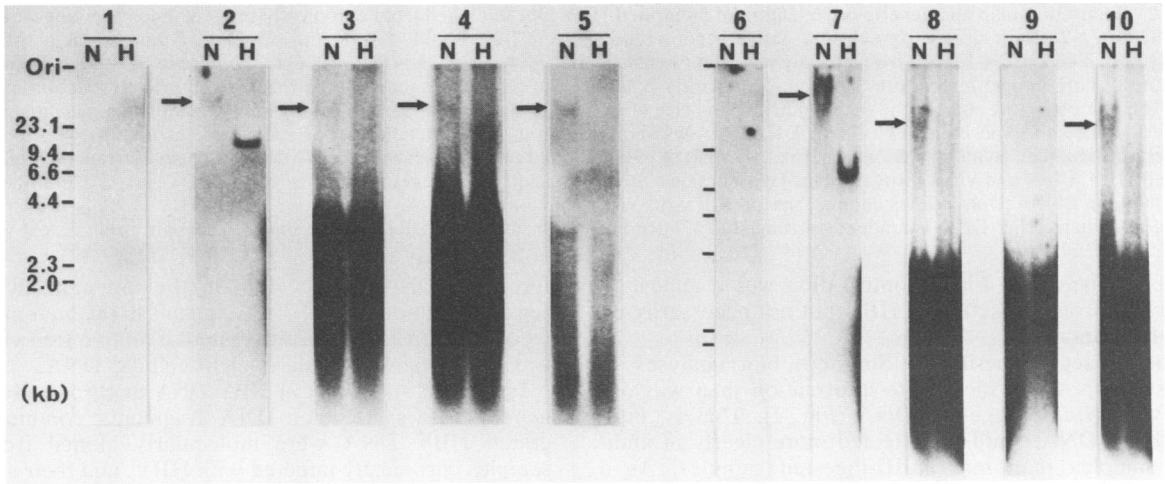

FIG. 1. Southern blot hybridization of undigested or *Hin*dIII-digested DNAs from chronically infected hepatic samples with an HBV DNA probe. DNA samples of 5 μg (lanes 1 to 8) or 7.5 μg (lanes 9 and 10) were electrophoresed on a 1% (lanes 1 to 5) or 0.8% (lanes 6 to 10) agarose gel and blotted on nitrocellulose paper. Lanes: 1 and 6, human thymus; 2 and 7, huH2-2; 3, NG; 4, NO; 5, NS; 8, T1; 9, T3; 10, T4. N, Nondigested; H, *Hin*dIII digested; Ori, origin.

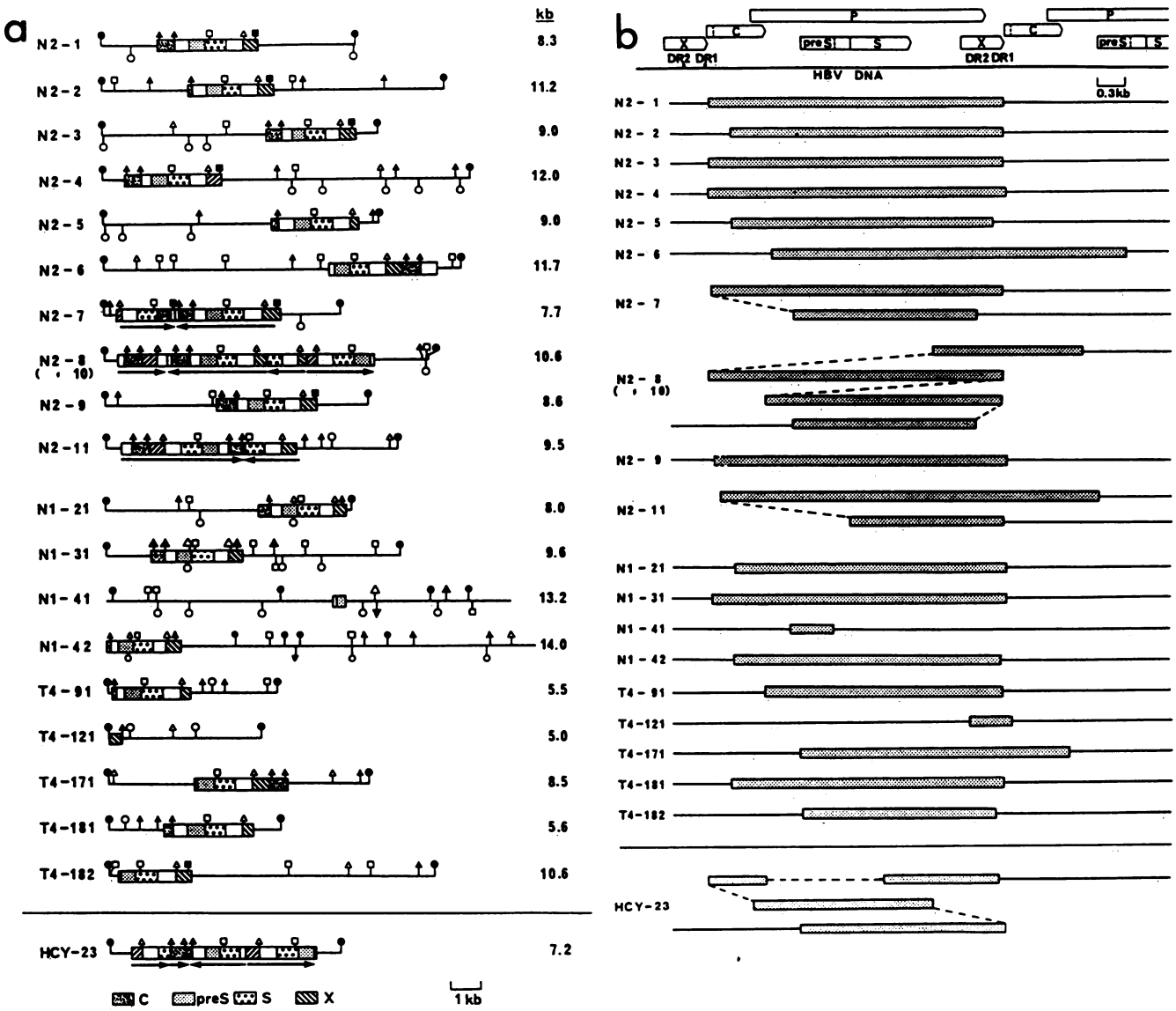

FIG. 2. Restriction map and genetic organization of integrated HBV DNAs cloned from chronically infected hepatic tissues and an HCC tissue. N2-1 to N2-11 are clones from chronic active hepatitis tissue N2. N1-21, N1-31, N1-41, and N1-42 are from chronic active hepatitis tissue N1 (33). T4-91 to T4-182 are from chronic persistent hepatitis tissue T4. HCY-23 is from HCC tissue. C, pre-S, S, and X represent the C (hepatitis B core antigen) gene, pre-S region, S (hepatitis B surface antigen) gene, and X gene, respectively. (a) Restriction maps of the clones. The number to the right of the figure indicates the size of each insert DNA of a clone. The boxed region indicates the integrated HBV genome, and the solid line indicates cellular DNA. Arrows show the viral DNA stretches in the rearranged structure, with the direction tentatively designated. Symbols: ●, *Hin*dIII; ○, *Eco*RI; ▲, *Bgl*II; △, *Bam*HI; □, *Xba*I; ■, *Rsa*I. (b) Schematic representation of the integrated HBV DNA and virus-virus or virus-cell junctions. Box represents the integrated regions of the HBV DNA. Solid lines indicate cellular flanking DNAs. Dotted lines connect the sites of virus-virus junctions. The gene organization of the HBV DNA is shown at the top of the figure, where HBV DNA is tandemly arranged at 1.7 genome length. DR1 and DR2 indicate the 11-base-pair direct repeat sequences.

Table 1 and lane 3 in Fig. 1), integration would appear to depend on chronic infection of HBV but not necessarily on drastic inflammation.

In the present experiment, Southern blot analyses of undigested DNA provided more information than was obtained with *Hin*dIII-digested DNA (Fig. 1). That is, integrated HBV DNA could be detected more clearly in undigested samples than in *Hin*dIII-digested samples. As a control experiment, the DNA from a blood sample from an HBV carrier whose blood contained HBV in high titer was examined. No hybridization signal was detected in the high-molecular-weight region, whereas an intense signal of free viral DNA was evident in the low-molecular-weight region (data not shown). Thus, signals in the high-molecular-weight region cannot be attributed at all to a free viral DNA trapped in high-molecular-weight cellular DNA.

Integrated structures of HBV DNA in chronically infected hepatic tissues. Cellular DNA fragments containing integrated HBV DNA were molecularly cloned from three samples chronically infected with HBV, and their structures were determined by restriction enzyme mapping and hybridization mapping with whole HBV DNA or gene-specific DNA fragments (11, 33) as the probe. The structures are shown in Fig. 2. Of 19 clones from N1, N2, and T4 DNAs, 12

carried colinear HBV DNA spanning from the DR1 region to the pre-S or C gene region through the X gene, as was often noted in previous reports on HCC samples (16, 33, 34). Two clones, N2-6 and T4-171, also had colinear HBV DNA, but both ends of the viral DNA were in the pre-S or its upstream region, possibly the second hot spot of recombination with cellular DNA or within viral DNA (16, 35; this work). In these two clones, the region corresponding to 0.8-kb X mRNA was conserved. We obtained three clones with gross rearrangement and inverted duplication of HBV DNA [N2-7, N2-8 or N2-10, N2-11]. This is the first direct evidence for the rearrangement of integrated HBV DNA from chronically infected hepatic tissues. Virus-virus junction was most frequently seen in the 5'-end region of the negative viral strand, and this was also true for the virus-cell junction. Two clones, N1-41 and T4-121, each containing a very small fragment of HBV DNA, were also obtained. To characterize these small viral DNA fragments, cellular flanking DNAs from clone N1-41 were assigned to chromosomes by using a human-mouse hybrid panel having 12 hybrid clones (data not shown). The left-side cellular flanking DNA was assigned to chromosome 4, while the right side was assigned to chromosome 6. It thus became clear that translocation occurred between cellular flanking DNAs. For clone T4-121, a left-side cellular flanking DNA probe was not available (Fig. 2a). The data indicated that rearrangement of HBV DNA or cellular DNA at the integration site was already present in all three samples chronically infected with HBV. It appears evident that rearrangement of HBV DNA as well as cellular flanking DNA is not specific for HCC cells.

Viral DNA rearrangement before integration. Analysis of virus-cell junctions of clone N2-7 by Southern blot hybridization indicated that both cellular flanking DNAs hybridized to a common cellular DNA fragment. For a more detailed examination, cellular counterpart DNA was cloned from the gene library of human thymus DNA by using cellular flanking DNA as the probe. Restriction enzyme mapping and sequence analysis of the cellular counterpart DNA demonstrated no gross change in cellular DNA except a small deletion of 30 base pairs at the integration site of inversely duplicated viral DNA (Fig. 3). Essentially the same was found from analysis of the clone HCY-23 from one HCC sample (HCY-23), in which a single copy of highly rearranged HBV DNA was integrated (Fig. 2 and 3). For HCY-23, 0.6 kb of cellular DNA was deleted. A similar structure has also been reported by Hsu et al. (6) in which highly rearranged WHV DNA was inserted 600 base pairs upstream of c-*myc* exon 1 with only a 3-base-pair cellular DNA deletion. Inverted duplication of HBV DNA thus possibly occurs before integration.

Limited sequence homology at virus-virus and virus-cell junctions. To provide some clarification of the mechanism of viral DNA rearrangement and also of that of integration to cellular DNA, we determined the sequence of junctions. At most virus-virus junctions, there is a one- to three-nucleotide identity between two viral DNA strands (Fig. 4, shaded areas). In such cases, one side of the adjoining viral sequences was found to be weakly homologous to each other (boxed in Fig. 4). Viral DNA rearrangement may have occurred by template switching or jumping of polymerase along with the 3' end of the nascent DNA strand to the complementary strand or within the same template through this patchy homology during the reaction. As for the virus-cell junctions (Fig. 5), a one- to five-nucleotide identity between viral and cellular DNAs was observed in all cases analyzed. In clones N2-1 through N2-4 as well as N2-21 and

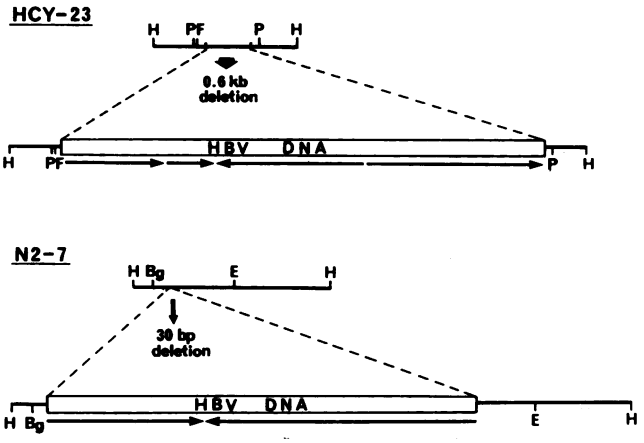

FIG. 3. Structure of integration sites before and after HBV DNA integration. Cellular counterpart DNAs of N2-7 and HCY-23 were molecularly cloned, and structures were analyzed by DNA sequencing (upper restriction maps in each figure). In both cases, no change except a small deletion at the junction site was found in cellular DNA. Restriction endonuclease sites are as follows: H, *Hin*dIII; P, *Pvu*II; F, *Hin*fI; Bg, *Bgl*II; E, *Eco*RI. bp, Base pairs.

N1-31, each cellular flanking DNA was found to be different based on restriction enzyme mapping (Fig. 2), indicating that all these clones were independent and not from a cloning artifact. Our previous data from HCC samples are also included in Fig. 5 (33, 34). Similar observations have been reported by other investigators (HBV [26], WHV [6], duck HBV [7]). From the data obtained, integration may occur basically by a mechanism similar to that for virus-virus junctions.

DISCUSSION

The present study demonstrated random integration of HBV DNA in the most chronically infected hepatic tissues by Southern blot analysis and provided direct evidence for viral or cellular DNA rearrangement in these tissues by molecular cloning and structural analyses. Random integration and rearrangement are shown here to occur in the early period of infection. No significant difference between chronic hepatitis and HCC cases could be found with respect to the integrated structure of HBV. Rearrangement of viral DNA or cellular DNA at the integration site is not a specific event in HCC cells. Thus, rearrangement at the site of HBV DNA integration is not sufficient for hepatocarcinogenesis. This possibility is supported by data from one hepatoma cell line containing a single copy of integrated HBV DNA without viral or cellular DNA rearrangement (34) and from a hepatoma tissue bearing multiple integration, none of which were rearranged, as determined by molecular cloning (33). Rather, DNA rearrangement in each tissue appears to be due to the individual characteristics and conditions of the host or viral replication, since its frequency is totally independent of whether the sample is from HCC or chronically infected hepatic tissue (16, 33; this work). A comparison of HCC 1707 (33) and N1 (33; this work) gives findings of interest. Southern blot patterns and integrated structures of HBV from these siblings are almost the same; however, the elder sibling developed a tumor but the younger has not, at least so far. This difference may depend on whether the second or third event subsequently occurs after HBV integration. A common feature throughout HCCs may be the integration

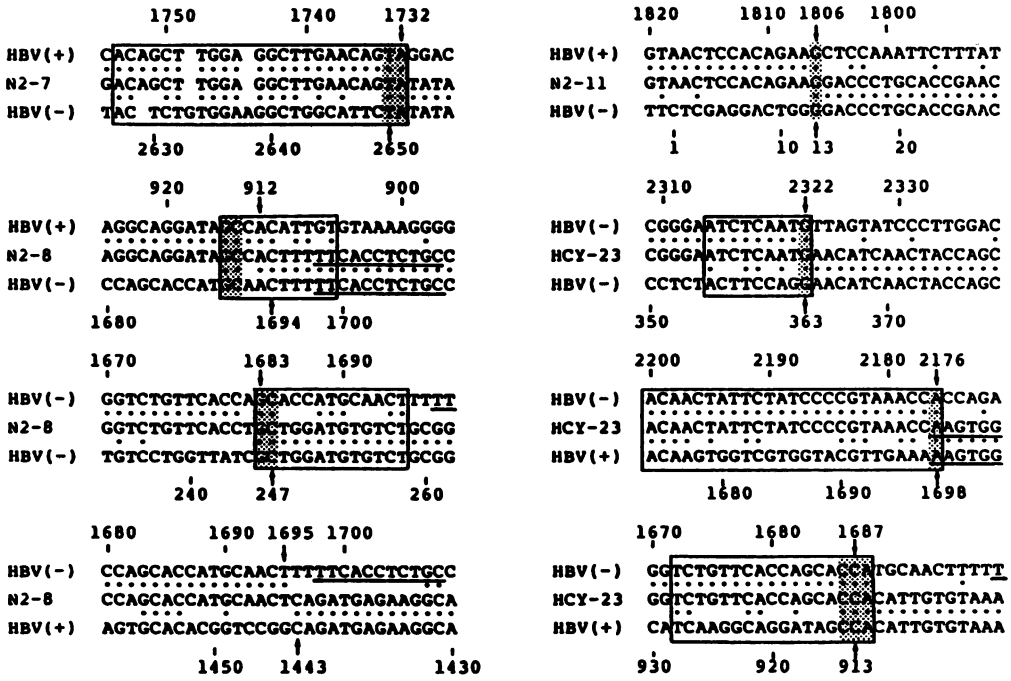

FIG. 4. Nucleotide sequences of virus-virus junctions. Dots denote nucleotides common to the plus or minus strand of HBV DNA and the clones. Nucleotide numbers of HBV DNA (subtype adr) (8) are shown above or below the nucleotide sequences. Shading shows identical nucleotides between two viral strands joined together. Underlines indicate DR1 sequence in the HBV genome. Weakly homologous regions adjacent to the virus-virus junctions are boxed.

itself, especially of the X gene as well as the envelope gene regions. This is perhaps the case in chronic hepatitis. Many HCCs bear at least one copy of such an HBV gene-containing integrant (11).

Based on the sequence data on virus-virus junctions, inverted duplication of viral DNA may be reasonably considered to occur by template switching or jumping of polymerase along with the 3′ end of the nascent DNA strand to the complementary strand or within the same template, respectively, by using a patchy homology on one side of adjoining viral sequences during the reaction. As for the manner in which integration occurs, our data suggest that this viral DNA rearrangement occurs before integration. That is, already rearranged viral DNA integrates to cellular DNA in some cases, simply because N2-7 and HCY-23 are not likely to be produced by recombination between two distantly integrated HBV DNAs or by inverted duplication of one original integrant without gross change in cellular flanking DNA. Dejean et al. (5) proposed a model in which the head-to-head oligomer of HBV DNA integrates through specific recombination between direct repeats in the HBV DNA and the cellular DNA, followed by some deletion that causes a loss of one of these junctions. But this model would explain only a limited number of cases of rearranged viral DNA, and an additional event for reorganization after integration is required.

No novel form of HBV DNA has been found yet, but it is assumed to exist as in the WHV and ground squirrel hepatitis virus systems. Although the mechanism for this is not fully clarified, a reasonable assumption is that a novel form of HBV DNA integrates into cellular DNA through some illegitimate recombination between viral and cellular DNAs by using the one- to five-nucleotide identity and that this recombination would occur most frequently in the cohesive

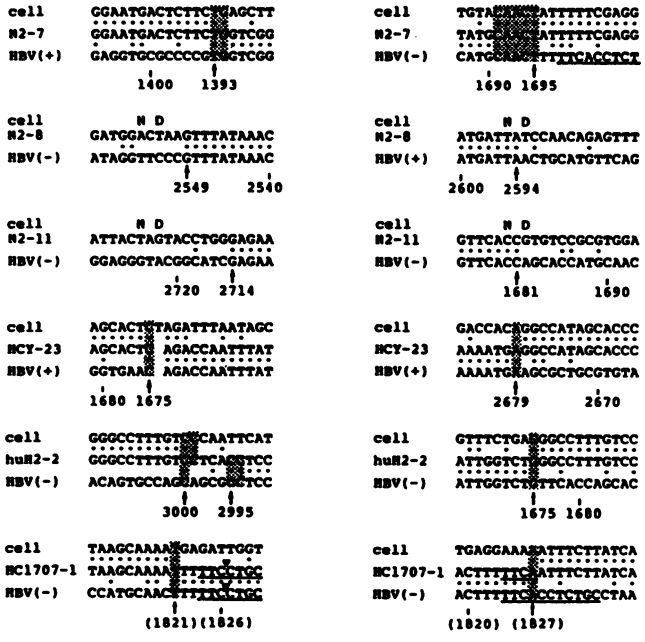

FIG. 5. Nucleotide sequences of virus-cell junctions. Dots denote nucleotides common to HBV DNA or cellular counterpart DNA and the clones. Nucleotide numbers of HBV DNA are as in Fig. 4. Shading shows identical nucleotides between viral and cellular sequences joined together. Underlines are DR1 sequences. huH2-2 and HC1707-1 are clones from an HCC cell line and HCC tissue, respectively (33, 34). Nucleotide numbers in HC1707-1 are in parentheses because they are from subtype adw (17). In the left-side junction of huH2-2, the sequence TCA of unknown origin is evident, but at either end of the cellular or HBV DNA, there is the common nucleotide sequence CC.

end region and possibly in the pre-S or its upstream region. These regions have also been noted by other investigators as a recombination hot spot (16, 35). Assuming integration by a novel form, the HCY-23 and N2-8 cases can be explained simply. Clones N2-6 and T4-171 without rearrangement can also be explained by this mode of integration. HBV integration is thus proposed to occur in two ways, each independent of the other: integration of the minus- or plus-strand viral DNA of replicative intermediates (34) and integration of the novel form. Whether the rearranged nucleic acid is DNA or RNA in origin and how a circular novel molecule is formed are matters yet to be understood. Confirmation of the existence of a novel form of HBV DNA and examination of the mechanism of its synthesis warrant additional research.

Data from analyses of integrated HBV DNAs performed on chronically infected hepatic and HCC samples suggest the following functional characteristics. Since one virus-cell junction was close to the 5′ end of the negative viral strand (DR1), the major part of the X open reading frame and upstream sequences as well as the envelope gene region were retained (11). No particular structure of integrated HBV DNA has yet been found at a high frequency in HCC. It should thus be reasonable to consider the expression of a cellular gene(s) to be activated in a *trans*-acting manner through an increase in the HBV gene product(s) at the time of chronic infection. Recently, the X gene product was found to *trans*-activate homologous and heterologous transcriptional enhancers (24, 28) and to stimulate the growth of mouse NIH 3T3 cells (11, 27). It was also recently reported that transgenic mice that overexpress the HBV large envelope protein in their hapatocytes develop chronic liver cell injury (4). The function of HBV DNA integrants from chronically infected hepatic tissues should be examined in regard to these points.

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