

## Integrated Structures of Duck Hepatitis B Virus DNA in Hepatocellular Carcinoma

FUMIO IMAZEKI,<sup>1,2</sup> KATSUYUKI YAGINUMA,<sup>1</sup> MASAO OMATA,<sup>2</sup> KUNIO OKUDA,<sup>2</sup> MIDORI KOBAYASHI,<sup>1</sup>  
AND KATSURO KOIKE<sup>1\*</sup>

Department of Gene Research, Cancer Institute, Tokyo 170,<sup>1</sup> and 1st Department of Medicine, Chiba University School of Medicine, Chiba 280,<sup>2</sup> Japan

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The structure of integrated viral DNA in a hepatocellular carcinoma of a duck from Chi-tung county in China was analyzed. Three different clones of integrated viral DNA,  $\lambda$ DHS 6-1,  $\lambda$ DHS 6-2, and  $\lambda$ DHE 6-2, were obtained from the neoplastic portion of the liver by molecular cloning. One of the three clones,  $\lambda$ DHS 6-1, showed inverted repetition of integrated viral DNA with chromosomal flanking sequences. Another clone,  $\lambda$ DHS 6-2, showed a head-to-head configuration of the core and surface gene regions of duck hepatitis B virus (DHBV) DNA. The virus-chromosome junctions were often located near direct repeat 1 or 2 of DHBV DNA in three independent clones. Nucleotide sequences at the virus-virus junctions in two clones,  $\lambda$ DHS 6-1 and 6-2, indicated the possible rearrangement of chromosomal DNA and recombination of viral DNA. DHBV DNA appears to be integrated into the genome of hepatocytes in a manner similar to that of human and woodchuck hepatitis viruses. Thus, the duck system may serve as a useful animal model for the study of human hepatocarcinogenesis.

Duck hepatitis B virus (DHBV) is a hepatitis B-like virus first detected in birds from China and then in domestic ducks in the United States (14). A variety of liver diseases, including hepatocellular carcinoma, have been observed in ducks from Chi-tung county in China (20). The integration of viral DNA into the chromosomal DNA of hepatocellular carcinoma tissue has been detected by Southern blot hybridization (30) in human (2, 7, 9, 11, 23), woodchuck (27), and ground squirrel (13) hepatocellular carcinomas. Thus, this integration may be important to hepatocarcinogenesis, but the mechanism involved remains to be determined.

The structures of integrated viral DNA in human (3, 4, 10, 17, 24, 29, 31) and woodchuck (19) hepatocellular carcinomas, but not those in duck carcinomas, have been extensively studied by molecular cloning and DNA sequencing. This is the first report of the molecular structures of integrated DHBV DNA in a duck hepatocellular carcinoma.

### MATERIALS AND METHODS

**Molecular cloning.** The duck hepatocellular carcinoma tissue used in this study was the same as that designated duck no. 8 in our previous study (30). Chromosomal DNA was extracted from the neoplastic part of the liver, as described previously (8), and partially digested with *EcoRI*. DNA fragments of 10 to 20 kilobases (kb) were fractionated by electrophoresis on a 1.0% agarose gel. Charon 4A was used as the cloning vector for the *EcoRI* fragments. Ligation was performed at a total DNA concentration of 300  $\mu$ g/ml and a molar ratio of the vector to cellular DNA of 1:1. The ligated DNA was packaged in vitro by the method of Enquist and Sternberg (5). In vitro packaging lysates were purchased from Amersham. A total of  $2 \times 10^5$  plaques of recombinant phages were screened with <sup>32</sup>P-labeled DHBV DNA by the method of Benton and Davis (1).

This was followed by the molecular cloning of the tumor DNA, using a vector different from Charon 4A. Chromosomal DNA was completely digested with *SacI*, and frag-

ments of 5 to 12 kb were purified by preparative agarose gel electrophoresis and ligated with  $\lambda$ gtWES $\lambda$ B arm DNAs. The DNA concentration in the ligation mixture was 500  $\mu$ g/ml. The ligated DNA was packaged in vitro,  $80 \times 10^4$  plaques of recombinant phages were screened, and the positive plaques were analyzed.

**Restriction mapping and preparation of the subgenomic probe.** Restriction maps of cloned DNAs were determined by partial digestion or by a comparison of DNA fragments from single-, double-, and triple-enzyme digestions. Detailed maps were then prepared by analyzing fragments resulting from subcloning the DNAs into the cloning vector M13mp18, using *Escherichia coli* JM109 as the host. The restriction enzyme digests were electrophoresed on 1.0% agarose gels, blotted onto nitrocellulose by the method of Southern (25), and hybridized with <sup>32</sup>P-labeled DHBV DNA by nick translation (21).

Subgenomic fragments of DHBV DNA were prepared by digestion of cloned DHBV DNA (12) (provided by W. S. Mason, Fox Chase Cancer Institute, Philadelphia, Pa.) with the appropriate enzymes (Fig. 1). They were then subcloned into the vector M13mp18. <sup>32</sup>P-labeled subgenomic fragments were obtained by nick translation after purification of the insert DNA by polyacrylamide gel electrophoresis.

**DNA sequencing.** DNA sequences at virus-chromosome and virus-virus junctions in relevant clones were determined by the method of Maxam and Gilbert (15).

### RESULTS

**Southern blot analysis of integrated viral DNA.** To study the state of viral DNA in the liver, Southern blot hybridization of DNA extracted from neoplastic and nonneoplastic portions of the liver was performed. A high-molecular-weight signal was observed in the uncut DNA preparation of the tumor (Fig. 2, lane 1). Three discrete bands of 13.0, 7.9, and 7.0 kb were identified after digestion with *SacI*, which has no cutting site in DHBV DNA (Fig. 2, lane 2). This indicates the possible integration of viral DNA at three different chromosomal sites. Five discrete bands (12.0, 7.6,

\* Corresponding author.

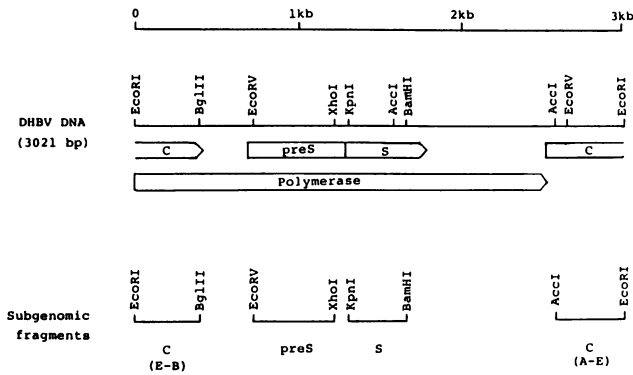


FIG. 1. Linear map of the gene organization of DHBV DNA (12). The major parts of the C-gene L fragment (*EcoRI*-*BglII*, 392 bp), pre-S (*EcoRV*-*XhoI*, 491 bp), the S gene (*KpnI*-*BamHI*, 365 bp), and the C-gene R fragment (*AccI*-*EcoRI*, 443 bp) were subcloned into the M13 vector and used as the gene probe to determine the structure of integrated DHBV DNA.

5.6, 4.4, and 3.9 kb) were seen after digestion with *EcoRI*, which cleaved DHBV DNA at one site (Fig. 2, lane 3). In addition to the integrated viral DNA, a large quantity of episomal viral DNA was identified in the neoplastic and nonneoplastic portions of the liver.

**Structures of integrated viral DNA.** Chromosomal DNA fragments of 10 to 20 kb resulting from partial digestion with *EcoRI* were cloned with a Charon 4A vector, resulting in the production of three different clones,  $\lambda$ DHE 4-1, 6-1, and 6-2. These clones may correspond to the three distinct bands indicated by arrows in Fig. 2, lane 3. However, each clone was found to have only one virus-chromosome junction, and the viral sequences at the other end were directly connected to the vector DNA in all three clones. To preserve both virus-chromosome junctions in the clone, chromosomal DNA fragments of 5 to 12 kb resulting from complete digestion with *SacI* were each cloned with a  $\lambda$ gtWES $\lambda$ B vector because of the cleavage site for *SacI* in DHBV DNA. Two independent clones,  $\lambda$ DHS 6-1 and 6-2, with virus-chromosome junctions on both sides were obtained. These two clones correspond to the two distinct bands indicated by arrows in Fig. 2, lane 2. Restriction mapping of  $\lambda$ DHS 6-1 and 6-2 indicated that they contain the same DNA fragments as those in  $\lambda$ DHE 4-1 and  $\lambda$ DHE 6-1, respectively, which were obtained by the previous cloning of *EcoRI*-digested chromosomal DNA (Fig. 3a and 3b). Thus, we were able to obtain three independent clones,  $\lambda$ DHS 6-1( $\lambda$ DHE 4-1),  $\lambda$ DHS 6-2( $\lambda$ DHE 6-1), and  $\lambda$ DHE 6-2, and their fine restriction maps were determined as follows.

A 7.9-kb *SacI* fragment was incorporated into clone  $\lambda$ DHS 6-1( $\lambda$ DHE 4-1); the restriction map of this fragment is shown in Fig. 3a. The fine restriction maps indicate that the viral sequences and chromosomal flanking sequences are extensively rearranged and a segment of DHBV DNA (S, pre-S, and C) is inversely repeated (Fig. 3a). Chromosomal flanking sequences at least 1.5 kb long are also inversely repeated. Using this flanking sequence as a probe, Southern blot hybridization was conducted with DNAs extracted from neoplastic and normal liver tissue. Two bands of 20.0 and 4.4 kb were seen in the *EcoRI*-digested tumor DNA (Fig. 4a). The former band was also evident in the *EcoRI*-digested nonneoplastic liver tissue and is considered to represent the counterpart of the homologous chromosomal DNA without viral DNA integration. The 4.4-kb band was seen with a flanking-sequence probe (Fig. 4a) and also with a DHBV

DNA probe (Fig. 2, lane 3). Thus, this band may represent a part of the homologous chromosomal DNA with integrated viral DNA. Since the signal at 4.4 kb appeared stronger than that at 20 kb (Fig. 4a), the 4.4-kb fragment with integrated viral DNA was probably amplified in this tumor. In addition, the signal of the *SacI* fragment with the flanking-sequence probe in the tumor (Fig. 4a, lane T) was also much stronger than that in the nonneoplastic liver tissue (lane N).

The restriction maps of a 7.0-kb *SacI* fragment from clone  $\lambda$ DHS 6-2( $\lambda$ DHE 6-1) are shown in Fig. 3b. The integrated viral DNA has a portion of the core gene and the surface antigen gene in a head-to-head configuration. By using the chromosomal flanking DNA of the right side of the 7.0-kb fragment as a probe, two bands at 7.0 and 5.6 kb were observed in the *SacI* digest of DNA from the tumor, whereas only the 5.6-kb band was noted in the *SacI* digest of normal liver DNA (Fig. 4b). The size of the integrated viral DNA in clone  $\lambda$ DHS 6-2 was approximately 2.5 kb, indicating that 1.1 kb of the chromosomal DNA may have been deleted at the integration site.

The restriction maps of the insert DNA in clone  $\lambda$ DHE 6-2 are shown in Fig. 3c. There was neither a large deletion nor rearrangement in the integrated viral DNA. The viral DNA was directly connected to the vector DNA on one side.

**DNA sequences of virus-chromosome and virus-virus junctions.** Small fragments containing virus-chromosome junctions

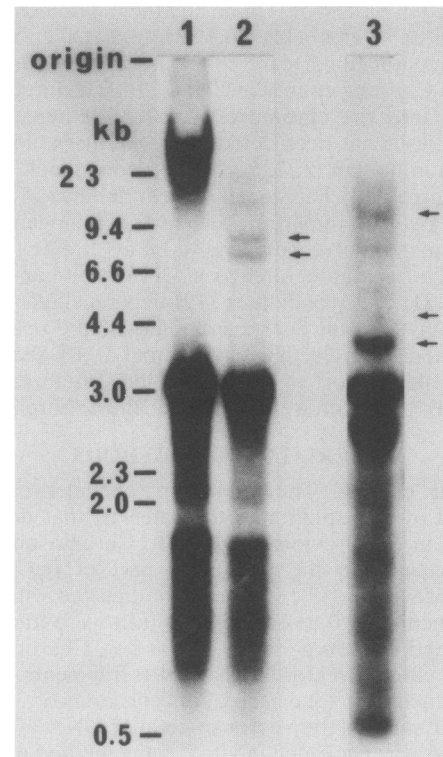


FIG. 2. Southern blot analysis of integrated DHBV DNA in a duck liver tumor. Chromosomal DNA extracted from a duck liver tumor was electrophoresed on a 1.0% agarose gel and analyzed by Southern blot hybridization with whole DHBV DNA as the probe. Lane 1, Undigested; lane 2, digested with *SacI*; lane 3, digested with *EcoRI*. Three discrete high-molecular-weight bands (13.0, 7.9, and 7.0 kb) can be seen in lane 2, and five bands (12.0, 7.6, 5.6, 4.4, and 3.9 kb) can be seen in lane 3. Arrows indicate the DNA fragments molecularly cloned. *HindIII*-digested lambda DNA fragments were run as size markers.

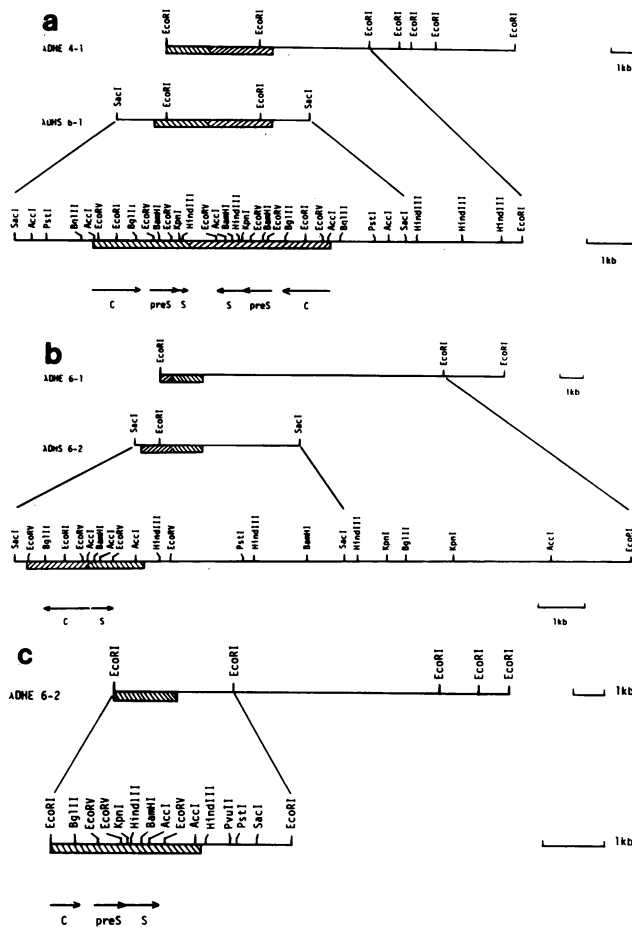


FIG. 3. Restriction endonuclease maps of *SacI* fragments from clones  $\lambda$ DHS 6-1 and  $\lambda$ DHS 6-2 and *EcoRI* fragments from clones  $\lambda$ DHE 4-1,  $\lambda$ DHE 6-1, and  $\lambda$ DHE 6-2. The duck cellular sequences are shown as thin lines, and the viral sequences are shown as hatched bars. The coding frames of the C gene, pre-S, and the S gene of the DHBV genome are shown at the bottom; arrows represent the 5'-to-3' direction(s) of the viral plus strand. (a) The 7.9-kb *SacI* fragment from  $\lambda$ DHS 6-1 and the *EcoRI* fragment from  $\lambda$ DHE 4-1; (b) the 7.0-kb *SacI* fragment from  $\lambda$ DHS 6-2 and the *EcoRI* fragment from  $\lambda$ DHE 6-1; (c) the 3.9-kb *EcoRI* fragment from  $\lambda$ DHE 6-2.

tions derived from  $\lambda$ DHS 6-1 DNA after *AccI-BglIII* digestion, from  $\lambda$ DHS 6-2 DNA after *AccI-HindIII* digestion, and from  $\lambda$ DHE 6-2 DNA after *AccI-HindIII* digestion were isolated and sequenced by the method of Maxam and Gilbert (15). The nucleotide sequences of the virus-chromosome junctions are shown in Fig. 5 and compared with the corresponding regions of the DHBV DNA sequence(s) reported by Mandart et al. (12). The virus-chromosome junction was 16 nucleotides downstream from direct repeat (DR) 1 in  $\lambda$ DHS 6-1, 5 nucleotides upstream from DR 1 in  $\lambda$ DHS 6-2, and 7 nucleotides downstream from DR 2 in  $\lambda$ DHE 6-2.

Fragments containing virus-virus junctions, derived from clone  $\lambda$ DHS 6-1 after *HindIII-EcoRV* digestion and from clone  $\lambda$ DHS 6-2 after *AccI-BamHI* digestion, were sequenced; the nucleotide sequences of the virus-virus junctions in the two clones are shown in Fig. 6. Six nucleotides in  $\lambda$ DHS 6-1 were found at positions 1345 to 1350 in the plus-strand sequence of DHBV (TGTTGA) and at positions 2506 to 2511 in the minus-strand sequence (TGTGGA) with one base change. Similarly, six identical nucleotides

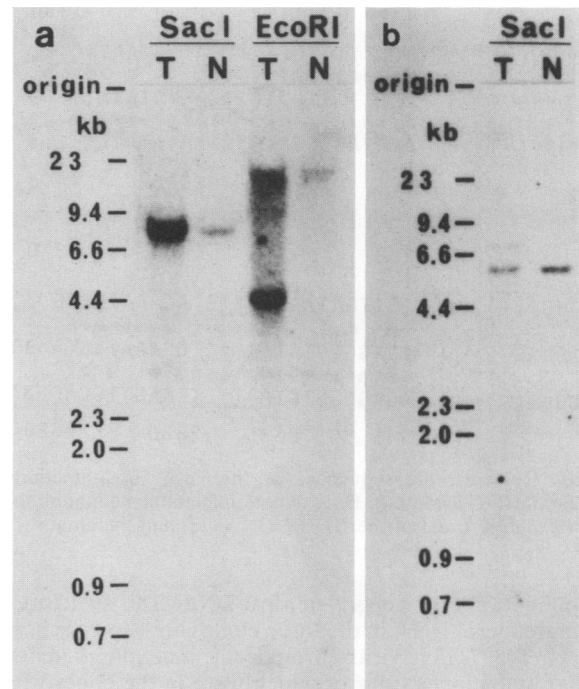


FIG. 4. Southern blot hybridization of DNA extracted from a liver tumor (T) and normal liver (N). DNA (10  $\mu$ g) digested with *SacI* and *EcoRI*, electrophoresed on a 1.0% agarose gel, and blotted onto a nitrocellulose filter. (a) A 1.0-kb flanking DNA fragment derived from *BglIII-AccI* digestion of  $\lambda$ DHS 6-1 (Fig. 3a) was labeled with  $^{32}$ P and used as the hybridization probe. *HindIII* fragments of lambda DNA were run as size markers. (b) Hybridization was performed using a 2.0-kb flanking DNA fragment derived from *HindIII* digestion of  $\lambda$ DHS 6-2 as the probe.

(AATTCT) in  $\lambda$ DHS 6-2 were detected at positions 1355 to 1360 in the plus-strand sequence of DHBV and at positions 2530 to 2535 in the minus-strand sequence. This suggests that virus-virus junctions, switches from a plus to a minus strand or vice versa, are formed in areas where there is at most six-nucleotide homology between the plus and minus strands of DHBV DNA.



FIG. 5. Nucleotide sequences of virus-chromosome junctions in clones  $\lambda$ DHS 6-1,  $\lambda$ DHS 6-2, and  $\lambda$ DHE 6-2. The nucleotide sequences were compared with the corresponding region(s) of DHBV DNA sequences reported by Mandart et al. (12). The sequence homologous to the DHBV DNA sequence is underlined in each clone.

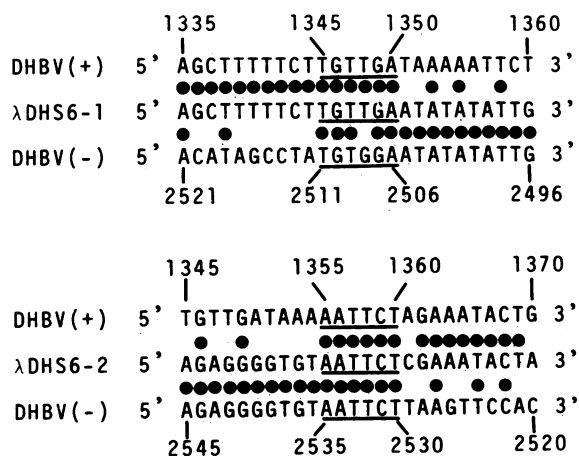


FIG. 6. Nucleotide sequences of the virus-virus junctions in clones  $\lambda$ DHS 6-1 and 6-2. Dots denote nucleotides common to the plus or minus strand of the DHBV DNA (12) and the clones.

**Configuration of integrated viral DNA.** The structures of integrated viral DNA in the three clones are shown schematically in Fig. 7. Five virus-chromosome junctions were found in the three clones, four of which were in the cohesive end region or just outside the DR. The remaining junction in clone  $\lambda$ DHS 6-2 was in the pre-S region. Two virus-virus junctions were found in clones  $\lambda$ DHS 6-1 and 6-2 (Fig. 7). Both showed similar configurations in which one junction was in the cohesive end region and the other was in the surface gene region.

## DISCUSSION

We molecularly cloned and analyzed three independent structures containing DHBV sequences integrated into the genomic DNA of the neoplastic portion of duck liver. Analysis of the clones indicated that none of the intact full-length DHBV genome was integrated into the chromosomes of duck liver; this has also been noted in human and woodchuck tumors (3, 4, 10, 17, 19, 24, 29, 31). Two clones,  $\lambda$ DHS 6-1 and  $\lambda$ DHE 6-2, had the entire region of the S gene, but none of the three clones had intact core and polymerase gene regions, suggesting that only surface protein is synthesized by integrated viral sequences.

In clone  $\lambda$ DHS 6-1, the integrated viral DNA was inversely duplicated with flanking chromosomal sequences (Fig. 3a), as previously reported for human hepatocellular carcinoma (17). This means that the same junction was present at both ends of the integrated viral DNA. This inversely repeating flanking sequence was at least 1.5 kb long and was found to be amplified several times with the viral sequence in the duck liver tumor (Fig. 4). In clone  $\lambda$ DHS 6-2, the C and S genes of the viral DNA were rearranged in a head-to-head configuration (Fig. 3b). These clones exhibited deletion or rearrangement of cellular sequences at viral integration sites, as has been reported for human hepatocellular carcinoma (22).

Virus-virus junctions in two clones,  $\lambda$ DHS 6-1 and 6-2, revealed some intriguing features. They were present in the cohesive end and S-gene regions and were connected to each other in opposite orientations (Fig. 7). Six nucleotides at the recombination site could be obtained from either of the two DHBV DNA strands (Fig. 6). Although this kind of regularity at virus-virus junctions has not been reported in humans

and woodchucks, it appears reasonable to speculate that switching occurs by abnormal replication in the region in which there is some nucleotide homology between the plus and minus strands of DHBV DNA.

All the virus-chromosome junctions except one were located in the vicinity of the cohesive end region between DR 1 and DR 2. DR 1 and DR 2 are each composed of 12-base-pair (bp) repeats (TACACCCTCTC) and start at nucleotides 2535 and 2477, respectively, from the *EcoRI* cutting site of DHBV DNA (12). Dejean et al. (4) showed one end of the integrated viral DNA segment to be in the vicinity of a DR. The mechanism of viral DNA integration into genomic DNA remains to be clarified, but it is conceivable by analogy to retroviruses (16) that hepatitis virus DNA is integrated at DRs. The model for viral DNA integration proposed by Yaginuma et al. (29), in which the 5' end of the minus-strand HBV DNA synthesized from the postulated plus-strand RNA pregenome (26) is inserted into chromosomal DNA, seems compatible with clones  $\lambda$ DHS 6-2 and  $\lambda$ DHE 6-2 but not with clone  $\lambda$ DHS 6-1, in which chromosomal DNA was linked to the region downstream from DR 1. At least one of the virus-chromosome junctions in human and woodchuck hepatocellular carcinomas is often situated in the cohesive end region between DR 1 and DR 2. However, chromosomal DNA is linked to the C gene in a few instances (4, 10). Since it appears likely that HBV DNA is integrated into the genome of hepatocytes before a hepatocellular carcinoma develops (28), the molecular structure of integrated DHBV DNA should be analyzed in the early phase of infection, i.e., chronic hepatitis, to gain a clear understanding of the mechanism of viral integration.

DHBV belongs to the hepadnavirus group and has the same structure as that of other mammalian viruses. However, one known difference is the absence of the X gene in DHBV DNA. Although the function of this gene is not known, antibodies against the translation product of this region have been detected in the sera of patients with

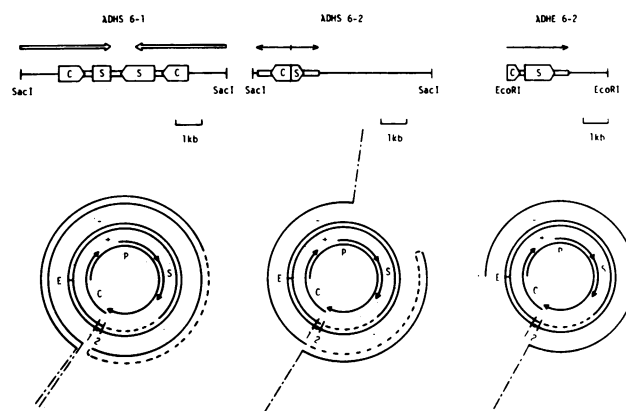


FIG. 7. Linear and circular maps of clones  $\lambda$ DHS 6-1,  $\lambda$ DHS 6-2, and  $\lambda$ DHE 6-2 and the orientations of viral DNA sequences. (Top) The inversely repeating regions oriented in opposite directions at both ends are indicated by boxes, and the other viral DNA sequences are indicated by bars. The thin lines represent cellular flanking DNA sequences. Parts of the C and S genes in a head-to-head configuration are shown by arrows. (Bottom) Maps of viral sequences in clones relative to the circular map of DHBV. The open reading frames of the S, C, and polymerase (P) genes are shown as the center circle, and the arrowheads indicate the 5'-to-3' direction of the viral plus strand. E, *EcoRI* site of DHBV DNA; 1 and 2, DR1 and 2, respectively.

hepatocellular carcinoma (18). The X-gene coding region and enhancer sequence were well conserved in most integrated HBV DNAs. Thus, the X gene-containing region may be closely related to hepatocarcinogenesis. In this regard, consideration may be directed to a relevant region (the carboxy-terminal region of the polymerase gene) of the DHBV genome.

Among four hepadnaviruses, various structures of integrated viral DNA in hepatocellular carcinoma have been reported for human hepatitis B (3, 4, 6, 10, 17, 22, 24, 29, 31) and woodchuck hepatitis (19) viruses. The present study showed that integration of DHBV DNA in the genome of hepatocytes is similar to that of human and woodchuck hepatitis virus DNA. Thus, the animal model used in this study may serve to demonstrate the importance of viral DNA integration in hepatocarcinogenesis.

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