JOURNAL OF VIROLOGY, June 1987, p. 1808–1813
0022-538X/87/061808-06$02.00/0

Vol. 61, No. 6

Multiple Integration Site of Hepatitis B Virus DNA in Hepatocellular Carcinoma and Chronic Active Hepatitis Tissues from Children

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Received 1 December 1986/Accepted 22 February 1987

Attention was directed to hepatitis B virus (HBV) integration in tissues obtained from an hepatocellular carcinoma (HCC) of an 11-year-old boy and from the liver of his 6-year-old brother, who had chronic active hepatitis. Multiple HBV DNA integration sites were demonstrated in both tissues. Cell population(s) in the HCC and liver from the patient with chronic active heptaitis were assumed to be heterogeneous with regard to HBV integration. The integrated forms in the two tissues showed similar genetic organization without gross rearrangement. The location of one of the virus-chromosomal junctions was restricted to the 5′-end region of the minus-strand DNA of HBV. The experimental results support our previous model for the mechanism of HBV integration, in which minus-strand replicative intermediates integrate into chromosomal DNA. The integrated HBV DNAs were conserved in the same region of the viral genome, spanning from the C gene through the S gene to the X gene, which contains intrinsic promoter-enhancer sequences.

Hepatitis B virus (HBV) infection causes acute and chronic hepatitis. Epidemiologic studies have shown a distinct correlation between the incidence of chronic HBV infection and the prevalence of hepatocellular carcinoma (HCC) (27). In addition, human chronic HBV carriers have an increased risk of HCC (1). The presence and integration of HBV DNA into the host genome of patients with HCC were demonstrated by Marion et al. (16) and at other laboratories (5, 6, 8, 24). The woodchuck hepatitis virus (WHV) induces HCC as a sequel to chronic WHV infection, and WHV DNA is also integrated into chromosomal DNA (19). Thus, HBV plays a major role in HBV-associated human HCC, and integration of the viral genome is often associated with development of the tumor.

Previous analyses have shown that subgenomic fragments of HBV DNA are integrated into the host chromosome with or without rearrangement of the viral genome (9, 10, 12, 13, 15, 18, 30). However, these data do not sufficiently clarify (i) the time of HBV integration following viral infection, (ii) the mechanism of HBV DNA integration, or (iii) the causal relation between HBV integration and the development of HCC.

To clarify some of these points, structural analyses were carried out on integrated HBV DNAs obtained from the HCC of an 11-year-old boy and from the liver of his 6-year-old brother, who had chronic active hepatitis, by molecular cloning and DNA sequencing. Essentially similar multiple patterns of HBV integration were observed in DNA from both HCC and chronic active hepatitis cases in which the genetic structure of integrated HBV DNAs corresponded to most, if not all, of the viral genome without rearrangement. One junction of the integrated HBV DNA was restricted to the 5′-end region of the viral minus strand, while the other junction was located at various sites. Terminal redundancy on both sides of the integrated viral sequence was also observed. The data from this study support our model for HBV DNA integration, in which minus-strand DNA of replicative intermediates that is synthesized by reverse transcription of the pregenomic RNA template is inserted into chromosomal DNA in the presence of defective nucleocapsid synthesis (29). Furthermore, direct evidence for the integration of HBV sequences into the DNA of patients with chronic active hepatitis prior to tumor development was obtained and is consistent with the indications presented previously by Brechot et al. (3, 4).

MATERIALS AND METHODS

Tissue samples. Tissue sample 1707 was obtained by autopsy from the HCC from an 11-year-old boy, whose serological markers were positive for hepatitis B surface antigen (HBsAg) and positive for antibodies to hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg) (28). The liver of his 6-year-old brother, who had chronic active hepatitis, was obtained surgically. The serum contained HBsAg, HBeAg, and the antibody to HBcAg. The sera and tissues of the two patients were analyzed for HBV markers, and tissue specimens were examined histologically (T. Morishima, K. Yaginuma, K. Matsuyuma, and K. Koike, manuscript in preparation). Both patients were infected with HBV of the same subtype adw, probably by their mother, a chronic HBV carrier. Tissue samples were immediately frozen in dry ice and kept at −70°C until DNA extraction.

Blot hybridization. Blot hybridization was performed by the method described by Southern (26). The ^{32}P-labeled hybridization probe of the HBV DNA or the chromosomal DNA fragment was made by nick translation (21).

Cloning and sequencing of the integrated HBV DNA. Chromosomal DNA was extracted by the sodium dodecyl sulfate-phenol method (28) from HCC sample 1707 or tissue from the patient with chronic active hepatitis and was completely digested with *Hin*dIII. DNA fragments from 7.6 to 20

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kilobases (kb) were fractionated by preparative agarose gel electrophoresis and ligated to a cloning vector, Charon 21AM, as described previously (29). After in vitro packaging, 5×10^5 plaques of each recombinant library were screened with a ^{32}P-labeled HBV DNA probe. Positive phages were selected by plaque hybridization (2), and their insert DNAs were recloned into the *Hin*dIII site of pBR322 DNA. No structural change in the insert DNA of each recombinant phage could be observed after it was recloned into pBR322.

Appropriate restriction fragments containing virus-chromosomal junction sites were isolated from each clone, and their nucleotide sequences were determined by the method described by Maxam and Gilbert (17).

RESULTS

Southern blot analyses. Chromosomal DNA was extracted from the tissue of HCC sample 1707, and the state of HBV DNA was analyzed by Southern blot hybridization. The hybridization of the undigested DNA exhibited no band in the low-molecular-weight region. In the *Hin*dIII digestion experiment, only diffuse hybridization from 7.6 to 20 kb was observed (Fig. 1, lanes a and b). HBV DNA was thus integrated variously into chromosomal DNA and was not present as free virus. Because discretely sized bands were observed in *Bam*HI and *Msp*I digestions, which cut the inside of the HBV genome, integrated sites of HBV DNA in the tissue of HCC sample 1707 were multiple in the chromosomal DNA. Some details regarding the Southern blot analyses of HCC sample 1707 DNA have been reported (28).

The total DNA of the liver of the patient with chronic active hepatitis was also analyzed by Southern blot hybridization. Chromosomal DNA was extracted by the sodium dodecyl sulfate-phenol method, omitting proteinase K treatment to reduce the amount of the viral DNA that was released from the interface. Hybridization of undigested DNA showed the presence of supercoiled DNA, and integration of HBV into chromosomal DNA was also observed (Fig. 1, lanes c and d). *Hin*dIII digestion presented a diffuse pattern of hybridization similar to that of HCC sample 1707, also indicating multiple populations of integrated HBV DNA. No distinct differences were found in hybridization patterns between tissues obtained from patients with HCC and chronic active hepatitis, except for the presence of free viral DNA in the latter case.

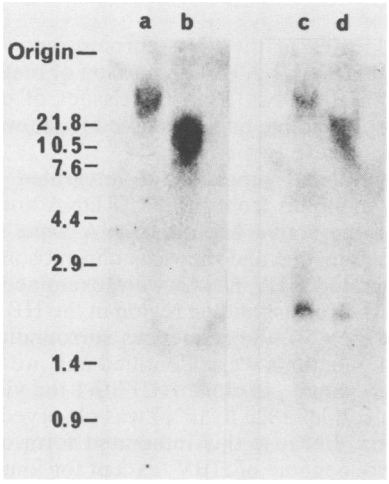

FIG. 1. Southern blot analyses of cellular DNAs extracted from tissues from HCC sample 1707 and a patient with chronic active hepatitis. Each chromosomal DNA sample (10 μg); which was undigested or digested with *Hin*dIII, subjected to 1% agarose gel electrophoresis, and blotted on nitrocellulose paper. As a hybridization probe, ^{32}P-labeled HBV DNA (subtype adr) (11) was used. Lanes a and b, undigested and *Hin*dIII-digested DNA of tissue from HCC sample 1707, respectively; lanes c and d, undigested and *Hin*dIII-digested DNA of tissue of a patient with chronic active hepatitis, respectively. Numbers to the left of the gel are in kilobases.

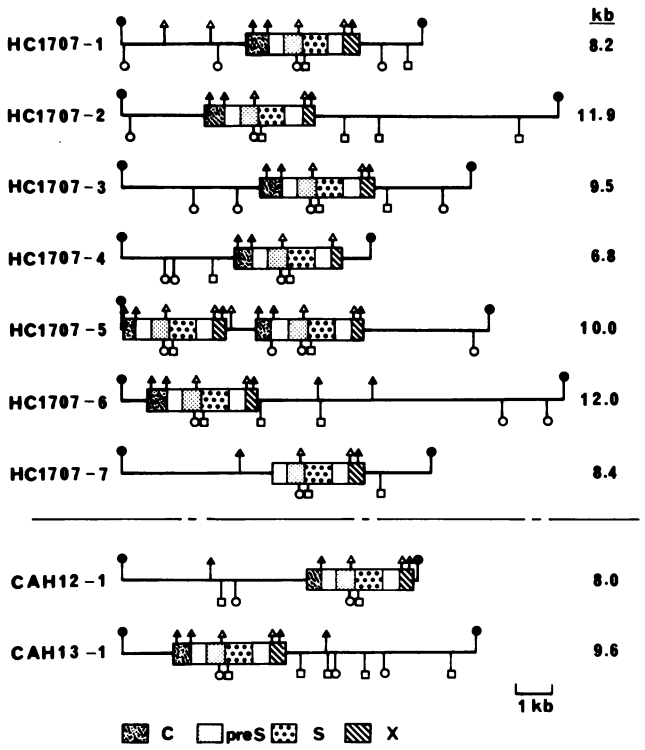

FIG. 2. Restriction maps and genetic organization of the integrated HBV DNAs cloned from tissues from HCC sample 1707 and a patient with chronic active hepatitis. HC1707-1 to -7 were cloned from the HCC sample 1707 tissue, and CAH12-1 and CAH13-1 were cloned from tissue of a patient with chronic active hepatitis. The numbers to the right of the figure indicate the size of each insert DNA of the clone. The boxed region indicates the integrated HBV genome, and the solid line indicates chromosomal DNA. X, S, preS, and C represent the X gene, S gene (HBsAg), preS region, and C gene (HBcAg), respectively. HBV DNA fragments of subtype adr (11) were subcloned at the *Eco*RI site of pBR322 with an *Eco*RI linker (13). The subcloned fragments corresponding to preS (nucleotides 2714 to 3109), the S gene (nucleotides 93 to 700), the X gene (nucleotides 1276 to 1645), and the C gene (nucleotides 1860 to 2299) were used as gene-specific probes. Symbols: ●, *Hin*dIII; ○, *Eco*RI; △, *Bam*Hi; ▲, *Bgl*II; □, *Xba*I.

Integrated structures of HBV DNA. Seven independent clones from the library of HCC sample 1707 DNA were obtained, denoted as HC1707-1 to -7, and were subjected to restriction enzyme and hybridization mapping analyses (Fig. 2). Six clones (all clones except HC1707-5) each contained a single copy of integrated HBV DNA. HC1707-5 contained two integrated forms of HBV DNA. Based on the restriction maps, the subtype of infected HBV DNA was found to be adw, and the structure of integrated HBV DNAs corresponded to the viral genome. Furthermore, results of hybrid-

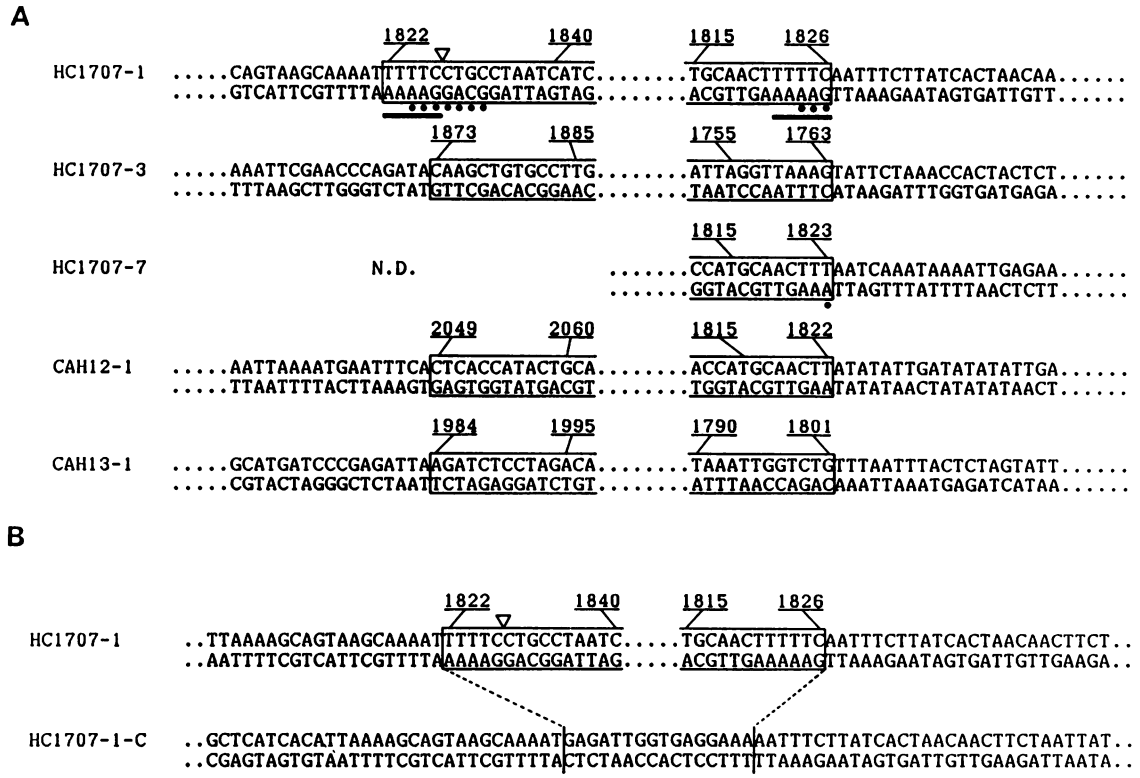

FIG. 3. Nucleotide sequences of virus-chromosomal junctions. (A) Junction sequences in the integrated forms of HBV DNA. Boxed regions represent the HBV sequences that were identified by a comparison with the corresponding region of the HBV genome of subtype adw (20). The numbers indicate the nucleotide position of the HBV genome of subtype adw. The open triangle indicates the deletion of ACCT (nucleotides 1827 to 1830). The dot denotes the nucleotides of the direct repeat sequence (DR1) The thick bar indicates the terminal redundant sequences. For the left end of the junctions in HC1707-7, the position was in the region between preS and gene C, but the nucleotide sequence has yet to be determined (N.D.). B. The nucleotide sequences of the virus-chromosomal junction in HC1707-1 DNA and its chromosomal counterpart DNA (HC1707-1-C). The 5.0-kb DNA fragment of the counterpart chromosome was cloned from the *Hin*dIII library of HCC1707 DNA by using chromosomal flanking sequences in clone HC1707-1 as a probe.

ization analyses with gene-specific probes showed that six clones contained the same region of viral genome, spanning from the C gene through the S gene to the X gene. Most, if not all, of the viral genome was integrated into the chromosomal DNA without gross rearrangement in the six clones. Only HC1707-7 failed to show hybridization with the C gene-specific probe, but it contained the region from the preS to the X gene. Results of these analyses present the unique and common features of integrated HBV DNAs in which two regions—one that is hybridized with the X gene-specific probe and the other that is hybridized with the C gene-specific probe—are directly connected to the chromosomal sequences. That is, the coding regions of the X gene and the C gene are located at two junction sites of each integrated form. In the case of HC1707-7, the preS region was located at the end opposite to the X gene. Thus, the data indicate that there is one restricted site for the integration of the HBV DNA molecule.

From the phage library of the DNA from the patient with chronic active hepatitis, two independent clones were selected and designated CAH12-1 and CAH13-1. These clones provided direct evidence for HBV integration in the 6-year-old patient with chronic active hepatitis, whose serological markers were positive for HBeAg and the antibody to HBcAg. From the results of the analyses of these two clones, it can be seen that almost the entire genome of HBV was integrated without rearrangment (Fig. 2). The X and C genes were located at the virus-chromosomal junctions of the integrated HBV DNA. A comparison of restriction maps of integrated HBV DNA from the tissues of patients with HCC and chronic active hepatitis failed to show any distinct difference(s) in these two cases.

Virus-chromosomal junctions of integrated HBV DNA. Three of seven clones from the HCC DNA and two clones from the chronic active hepatitis DNA were subjected to nucleotide sequencing, and the virus-chromosomal junctions of these integrated HBV DNAs were examined. By a comparison with the corresponding region in the HBV genome of the adw subtype (20), the sequences surrounding the virus-chromosomal junctions were identified and are presented in Fig. 3A. Interestingly, in clone HC1707-1 the viral sequence TTTTC (nucleotides 1822 to 1826) was observed to repeat at both junctions. Because this integrated form contained almost the entire genome of HBV, except for four nucleotides from 1827 to 1830, it may have a small terminal redundancy. To confirm this we also cloned the chromosomal counterpart of HC1707-1 DNA, denoted as HC1707-1-C, and analyzed the sequences at the integration site. The TTTTC sequence was derived from HBV and was terminally redundant (Fig. 3B). In contrast, however, the 16-bp chromosomal sequence was deleted as a result of HBV integration. In the case of HC1707-3, one junction was located at nucleotide 1763 and the other was at nucleotide 1873. A total of 110 bp was lacking from the full length of the viral genome. In clone

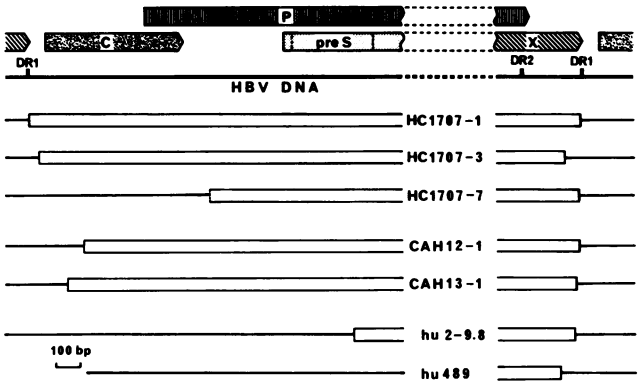

FIG. 4. Schematic representation of the genetic organization of the integrated HBV genome and virus-chromosomal junctions. Open boxes represent the integrated region of the HBV genome; solid lines indicate chromosomal flanking DNAs. The gene organization of the HBV genome is shown at the top of the figure. Abbreviations: X, X gene; preS, preS region; C, C gene (HBcAg); P, P gene. DR1 and DR2 indicate the 11-bp directly repeating sequences. Clones hu2-9.8 (29) and hu489 (18) are the integrated forms of HBV DNA from the HCC cells huH2-2 and hu-SP, respectively.

HC1707-7 the C gene was not present. One junction of HBV DNA was located at nucleotide 1823. The other junction was located in the region between the preS and the C genes.

The virus-chromosomal junctions of two clones from the tissue of the patient with chronic active hepatitis are also presented in Fig. 3A. In clone CAH12-1, one junction was at nucleotide 1822 and the other was at nucleotide 2049. In another clone, CAH13-1, the two junctions were at nucleotides 1801 and 1985, respectively.

One virus-chromosomal junction of each integrated HBV DNA was restricted exclusively to the region, along with the 11-bp direct repeat (DR1) upstream of the 3′ end of the X gene (Fig. 4). In particular, in the three clones HC1707-1, HC1707-7, and CAH12-1, the junction sites were very close to each other, at nucleotides 1826, 1823, and 1822, respectively. Our data indicate the presence of one fixed or preferred site of integration in the HBV genome. As for the other virus-chromosomal junctions, the site varied in the region from the C gene to the preS. From a comparison of the chromosomal flanking sequences at the integration site, no significant homology could be found.

To investigate changes in chromosomal DNA sequences by HBV integration, Southern blot analyses were performed, with the chromosomal flanking sequence from clone HC1707-1, HC1707-3, or CAH13-1 used as a probe (data not shown). In the case of HC1707-1, the original size of the *Hin*dIII fragment prior to HBV integration was estimated to be 5.0 kb. A comparison was conducted of this chromosomal counterpart (HC1707-1-C) with clone HC1707-1 by restriction enzyme mapping, but no rearrangement of chromosomal sequences could be observed. Thus, the results indicate that a full-sized genome of HBV (3.2 kb) was integrated with only a deletion of 16 bp at the integration site, as described above (Fig. 3B). In the case of HC1707-3, the size of the *Hin*dIII counterpart fragment was found to be about 2.5 kb. The 9.5-kb insert from clone HC1707-3 could not be explained simply by an insertion of 3.1 kb of HBV DNA (viral DNA with a 110-bp deletion) into this 2.5-kb *Hin*dIII fragment. Extensive deletion of chromosomal DNA must occur at the virus-chromosomal junction, when HBV DNA is integrated. Furthermore, in the case of clone CAH13-1 from the patient with chronic active hepatitis, an approximately 0.3-kb deletion of chromosomal DNA was observed at the virus-chromosomal junction. This finding indicates that deletion of chromosomal DNA sequences is not specific for the integration of HBV DNA in HCC.

DISCUSSION

Recently, Korba et al. (14) have shown that most woodchucks, which become chronic carriers following inoculation of WHV at birth, develop HCC. Some structural characteristics of an integrated form of WHV for chronically infected hepatocytes also have been reported (22). These reports stimulate an interest in a discussion about the state of integrated HBV DNA in HCC or in the livers of young patients with chronic active hepatitis based on our observations presented here.

When seven clones of integrated HBV DNA from HCC sample 1707 and two clones from the tissue of the patient with chronic active hepatitis were analyzed, essentially the common features of HBV integration could be observed in the HCC and chronic active hepatitis cases. One of the virus-chromosomal junctions was located exclusively in the 5′-end region of the viral minus-strand DNA. On the other hand, the other site of the virus-chromosomal junction varied within the region spanning from the C gene to the preS. In contrast to the results of a previous report (29), duplication of chromosomal flanking sequences at the integration site was not observed in these clones. This was considered to be due to the deletion of cellular sequences at the integration site, as shown in the case of clones HC1707-1, HC1707-3, CAH13-1, and others that have been reported previously (15). Thus, the unique features of HBV DNA integration are consistent with our proposed model of HBV integration, in which replicative intermediates are integrated into chromosomal DNA in the presence of defective nucleocapsid synthesis (29).

Because one of the virus-chromosomal junction sites was located at nucleotide 1826 in clone HC1707-1, which contained the largest integrated form of HBV DNA among all the clones, the 5′ end of the replicative intermediate of the minus-strand DNA may possibly be located at nucleotide 1826 or 1827. This is in good agreement with the location indicated by our model of HBV integration. It is also of considerable interest that the small terminal redundancy of TTTTC (nucleotides 1822 to 1826) was observed in integrated HBV DNA at the virus-chromsomal junctions in clone HC1707-1. Buscher et al. (7) have proposed the possible presence of the terminal redundancy in a replicative intermediate of the viral minus-strand DNA in their implication of reverse transcription of duck hepatitis B virus. Seeger et al. (23) have demonstrated such terminal redundancy in the case of ground squirrel hepatitis virus. The terminal redundancy in clone HC1707-1 is thus a direct indication that the integrated HBV DNA is quite likely the replicative intermediate that is synthesized by reverse transcription of the RNA pregenome. Dejean et al. (10) have proposed a homologous recombination model of HBV DNA integration, in which a specific recombination event within the direct repeat sequences may occur between viral and chromosomal DNAs. Nucleotide sequences of the chromosomal DNA at the junction sites, however, showed the absence of any significant sequence homology between viral and chromosomal DNAs. Therefore, the mechanism of recombination leading to HBV integration into chromosomal DNA is still obscure.

On comparison of HBV integration in the tissue of HCC sample 1707 with that in the liver of the patient with chronic

active hepatitis, a similar diffuse pattern of hybridization was observed by Southern blot analyses. The populations of HCC cells containing HBV DNA may thus possibly be heterogeneous. In fact, the copy number of the integrated HBV DNA per genome was estimated to be one to two on the basis of its hybridization intensity when compared with that of the c-H-*ras* sequence as the internal reference (unpublished data). Furthermore, various populations of integrated HBV DNA in tissues from children with HCC and chronic active heptatitis were proved by molecular cloning. These findings differ from the results obtained from cases of HCC in adult patients, in which blot hybridization with the HBV DNA probe showed some discrete bands. Although the incidence of childhood HCC is rare, a possible explanation for our data may be as follows. Many transformed hepatocytes, each with a different HBV DNA integration, may appear within a short period and grow as independent cell populations, without incurring exclusive reactions arising from host immunological responses. This probably brought about the polyclonal growth of the HCC sample 1707 cells rather than the monoclonal growth. Such a phenomenon may be a specific case that occurs especially in children, in which HCC progresses much more rapidly than in adult patients. A diffuse pattern of hybridization was also observed in three other unrelated children with chronic active hepatitis (unpublished data).

For clarification of the possible functions of integrated HBV DNA in the establishment of malignant transformation, much additional research must be carried out. However, based on the unique and common features of HBV DNA integration in children with HCC and chronic active hepatitis that have been examined so far, it is conceivable that integration of HBV may provide an HBV-related product in the state of chronic active hepatitis that is not sufficient but that is a main cause for the formation of HCC. Because one of the virus-chromosomal junctions of all integrated forms was found to be restricted to the region of the C terminus of the X gene (Fig. 4), there is the possibility of the expression of an X gene-related protein that may operate at the early step of a tumorigenic process. In addition, because all integrated forms of HBV DNA that were analyzed in our previous studies (13, 18, 29) and in this study included the intrinsic promoter-enhancer region identified by Shaul et al. (25). It is possible that expression of cellular oncogene(s) may be activated by this integrated promoter-enhancer sequence itself or through an increase in the HBV-related product at the early stage of tumorigenesis.

ACKNOWLEDGMENTS

We thank Nobuo Tsuchida and Jesse Summers for stimulating discussions. We also thank Takashi Sugimura and Hans Popper for suggestions and critiques of the manuscript.

This work was supported in part by a grant-in-aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control and by grants-in-aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

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