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HEPATITIS B VIRUS DNA INTEGRATION AND EXPRESSION OF AN ERB B-LIKE GENE IN HUMAN HEPATOCELLULAR CARCINOMA

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Southern blot studies on Hepatitis B Virus (HBV) DNA integration in 13 human hepatocellular carcinomas (HCCs) patients revealed the presence of several distinct HBV integration sites in different human liver disease patients. In one HCC patient the DNA fragment containing the HBV integration also hybridized to an erb B probe. The erb B/HBV co-migrating DNA fragment was cloned and sequenced, and showed that HBV DNA is integrated next to a cellular DNA fragment which is homologous to the tyrosine protein kinase domain of the human epidermal growth factor receptor gene and other cell surface receptor genes. The virus-integrated cellular DNA sequence is expressed in this HCC patient, suggesting a possible role for this gene in hepatocarcinogenesis.

Epidemiologic studies have shown a strong correlation between the incidence of chronic hepatitis B virus (HBV) infection and the prevalence of a wide variety of liver diseases such as acute hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) (1). The molecular basis of this link between HBV infection and hepatoma development has thus far remained elusive. Since HBV DNA is integrated into human liver DNA in almost all HCCs (2), and since the integration of viral DNA is not a required step in the replication cycle of the virus (3), the presence of integrated viral sequences in many HCCs is suggestive of a causal relationship between viral DNA integration and subsequent tumor development (4). In support of this viewpoint, HBV integration is often associated with chromosomal abnormalities (5-7), including deletion, duplication and chromosomal translocation. These extensive rearrangements of the cellular flanking sequences and rearrangement of the integrated HBV genome are suspected to give rise to some effects on cellular oncogenes and may play a part in liver carcinogenesis (3). In two woodchuck hepatitis virus-induced HCCs, viral DNA was found integrated in the c-myc gene and expression of the c-myc gene was activated (4,8,9). One group of researchers found that viral integration occured in the human retinoic acid receptor gene (10). Another group found a viral DNA integration on chromosome 17q near the p53 gene which is commonly deleted in HCC (11). However, the

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majority of HBV integrations cloned from HCCs have not been found to associate with any known cellular oncogenes or common cellular DNA sequences.

In order to examine the possible molecular mechanisms by which HBV integration may lead to the development of the transformed phenotype, we examined HCC cases of Chinese patients for HBV DNA integration. We describe here the presence of several distinct HBV integration bands in different individuals. One of these integration bands contains a DNA sequence with homology to the tyrosine protein kinase domain of the human epidermal growth factor receptor gene, which was found to be expressed in the HCC patient.

MATERIALS AND METHODS

Human tissue

Human hepatoma, normal adult and cirrhotic liver specimens were obtained at the time of surgical resection at the Shanghai Institute of Digestive Disease, Shanghai Second Medical University, Shanghai, China. The surgical specimens were stored at -70°C until analyzed.

Isolation and blotting analysis of genomic DNA

Genomic DNA was extracted from the tissue according to Zhang et al. (12). Briefly, tissues were homogenized in 25 Mm Tris, pH 8.0, 100 Mm NaCl, 1 Mm EDTA and 0.6% SDS buffer, and then incubated with proteinase K (0.7 mg/ml) at 37°C overnight. Following extraction with phenol and then chloroform-isoamyl alcohol (24:1), the DNA was precipitated with 2.5 vol ethanol. The purified DNA was digested with restriction endonucleases (BRL laboratory) under conditions recommended by the manufacturer. The digested DNA samples were separated on 1.0% agarose gels, and transferred to nitrocellulose filters. Filters were then hybridized with DNA probes radiolabeled by nick-translation as described elsewhere (12).

RNA isolation and blot hybridization

Total cellular RNA was isolated from the liver tissue specimens using the isothiocyanate procedure (13) with modification (14). The isolated RNA was then spotted onto a nitrocellulose filter and hybridized with a ^{32}P-labeled DNA probe as previously described (14).

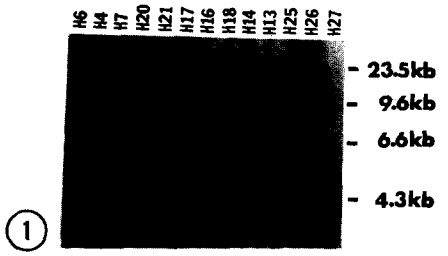
Cloning and sequencing

Genomic DNA from liver tissue of patient H4 was digested with Hind III and fractionated on an agarose gel. The DNA fragments corresponding to the 5 kb range were purified by electroelution, and subsequently cloned into plasmid pGEM-7Zf(+) (Promega, Madison, WI). The cloned DNA were screened with a ^{32}P-labeled 3.2 kb HBV DNA probe and a 1.7 kb erb B DNA probe. Following isolation of a positive clone, deletions were constructed using the Erase-a Base System (Promega) and subcloned in pGEM-7Zf(+). These subclones were then alkaline denatured and sequenced using the dideoxy sequencing method (15) with the sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and primers complementary to T7 or SP6 promoters in plasmid PGEM-7Zf(+).

RESULTS

Integration state of HBV DNA in human liver disease patients

To determine the state of HBV integration 13 cases of HCC which were diagnosed as hepatitis B surface antigen-positive, were examined by Southern



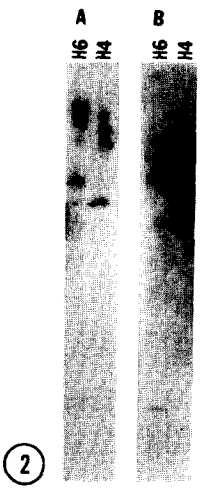


Figure 1. The HBV integration state of human hepatoma. Genomic DNAs were prepared from human HCC tissues (H) and digested with restriction enzyme Bam Hl. The digested DNAs were blotted and hybridized with 3.2 kb HBV cloned DNA. Arrows and stars indicate the HBV integration fragments present in more than two different individual patients.

Figure 2. Southern Blot analysis of HBV integration in hepatoma patient H4 and its relation to the erb B-like gene structure. Genomic DNA from HCC patients was digested with Bam HI and hybridized with 3.2 kb HBV probe (A). The nitrocellulose filters were then strip washed and rehybridized with a 1.7 kb erb B DNA probe (B).

blot analysis. Figure 1 shows the Southern blot analysis with Bam Hl digested DNAs from these patients hybridized with a ^{32}P-labeled 3.2 kb cloned HBV DNA. Hybridizing bands of DNA of sizes greater than the 3.2 kb size of fully double stranded HBV DNA in all HCC patients demonstrated that the viral sequences were integrated into the cellular genome in these patients. All normal human liver and three HCC DNAs did not show any hybridization signal (data not shown). The comparison of HBV hybridization patterns of different patients revealed the existence of discrete bands of size greater than 3.2 kb, suggesting that HBV sequences were integrated into cellular DNA in these patients.

Co-migration of HBV DNA with an erb B-like sequence

In our previous communication (16), we showed the structural alteration of the c-erb B oncogene in human liver cirrhosis and hepatomas. In order to study whether this altered structure is caused by HBV integration, DNA samples digested with Bam Hl were hybridized with radioactively labeled erb B DNA. As shown in figure 2, when the nitrocellulose filter used to hybridize with labeled HBV DNA is rehybridized to a 1.7 kb erb B DNA probe which represents the v-erb B gene of avian erythroblastosis virus strain H (17), two DNA fragments were detected in patient H4. Patient H6 is shown here as a representative of patients who do not have this additional band. This

fragment was found to co-migrate with HBV DNA by comparison of the hybridization patterns of Figure 2A and 2B, suggesting that this DNA fragment may represent a DNA fragment containing both HBV and erb B sequences.

To exclude the possibility that HBV and erb B coincidentally hybridized to different fragment with the same molecular weight, the co-migration of HBV and erb B DNA sequence in patient H4 was further studied by Southern blot studies with other restriction enzymes. The HBV/erb B co-migrating fragment in patient H4 was also detected when Hind III, Eco RI and Pstl were used (data were not shown). Therefore, the comparison of the HBV hybridization pattern and that of erb B using different restriction enzymes strongly suggests that HBV was integrated in or very near to a cellular DNA sequence similar to the erb B gene.

HBV is integrated in an erb B-like sequence

In order to examine in more detail the nature of the erb B-like gene association, especially with respect to HBV DNA integration, the Hind III digested genomic DNA of patient H4 was electrophoresed through an agarose gel, and the DNA fragment containing both HBV and erb B-like sequences was excised, purified and subsequently cloned in plasmid pGEM-7Zf(+). Colonies were selected for their hybridization to HBV DNA and erb B DNA probes. One of these clones (designated as H4erb) was subjected to further analysis.

By restriction analysis and subsequent blot hybridization with sub-genomic viral DNA probes, we determined that the viral insert consists of a continuous sub-genomic fragment of 2.9 kb (Figure 3). To study the integration behavior in more detail, the cloned H4erb DNA was sequenced. The integration site falls within the single-stranded region of the HBV genome, suggested to be a preferred HBV integration region (18,19). HBV integration

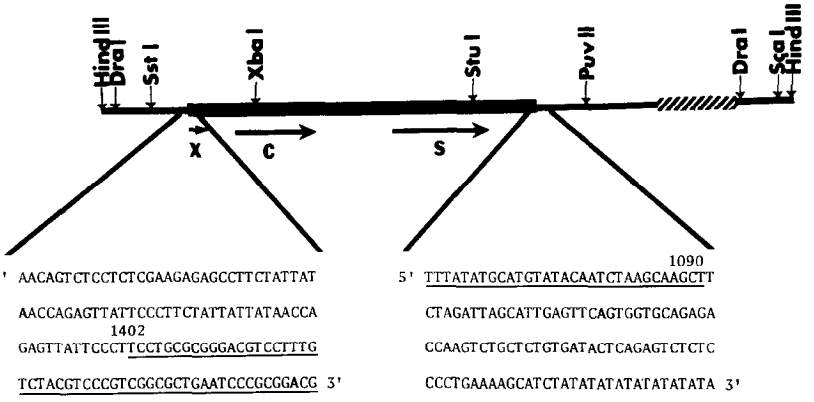


Figure 3. Restriction map of the cloned H4erb DNA and nucleotide sequence of viral and cellular DNA junctions. The HBV DNA sequence, which is underlined, starts from position 1402 and ends at position 1090. HBV S gene, C gene, and X gene are indicated as S, C, and X, respectively. The hatched box of cellular flanking sequence indicates the region homologous to the erb B gene.

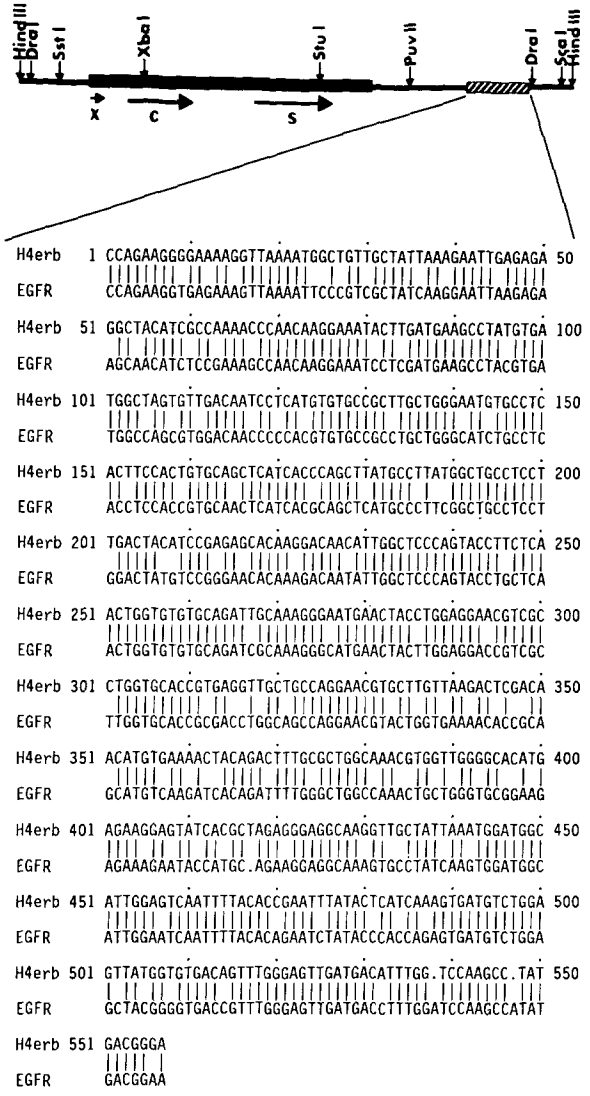


Figure 4. Sequence comparison of the 550 bp cellular flanking sequence of cloned H4erb DNA with the human EGF receptor gene. The map at the top of the figure is the same as that described in Figure 3.

occurred within the X gene, starting at position 1402 bp and terminating at position 1090 bp. This fragment contains the entire C and S gene and the 3' half of the X gene. About 30 bp and 300 bp were deleted from the X gene and P gene respectively. The breakpoint was 188 bp from the direct repeat which is believed to be a preferred HBV integration site. Our sequence data also confirmed that the integrated viral S gene is close to a cellular erb B-like sequence. The sequence data also reveals that 550 bp of cellular DNA sequence which is about 1 kb away from the integration site shared 83% identity with the tyrosine protein kinase domain of the human epidermal growth factor (EGF) receptor gene (20) (Figure 4). This sequence is also homologous to the human c-erb B-2 gene (73% identity) and other cell surface receptor genes (21,22).

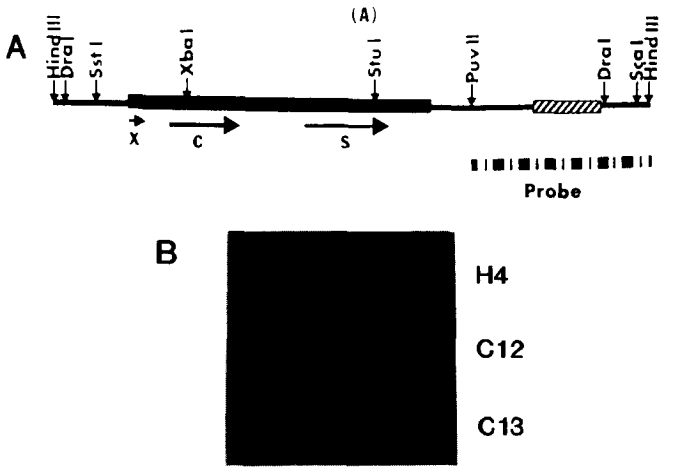


Figure 5. The expression of erb B-like sequence in hepatoma patient H4. (A) shows the 1.2 PvuII/HindIII flanking cellular DNA fragment used as a hybridization probe to study gene expression. (B) Expression of the cloned erb B-like gene in normal human liver and hepatoma patient H4. RNA isolated from patients' liver or hepatoma tissue was spotted onto a nitrocellulose filter and hybridized with a ^{32}P-labeled 1.2 kb flanking cellular DNA fragment.

Expression of integrated cellular sequence

To study if this integrated erb B-like sequence is expressed, the 3'-flanking cellular sequence was isolated. This 1.2 kb Pvu II fragment was labeled and used as a probe to hybridize to RNA samples from human liver disease patients. As shown in Figure 5, this sequence is not expressed in normal human liver samples (C12 and C13) while it is clearly expressed in patient H4.

DISCUSSION

The integration of HBV in both HCC derived cell lines and human tumors has been found to be in a random fashion as determined by Southern blotting (18, 23-27). In the present study, we observed the presence of a distinct HBV integration fragment which also hybridized with a viral erb B DNA probe. The mechanisms by which HBV contributes to the malignant process in liver cells is not known. The HBV sequence could act as an insertional cis-acting promoter-/enhancer that activates nearby cellular genes (2). HBV integration in a retinoic acid receptor gene has been reported (10). More recently, woodchuck hepatitis virus DNA was found to have integrated into the c-myc gene in two cases of HCC, resulting in altered expression of this gene (4). We offer evidence here that HBV DNA is integrated in a region of the human genome that is similar to the cellular oncogene encoding the receptor for epidermal growth factor. The integrated cellular sequence shows a very high degree of similarity to the tyrosine protein kinase domain of human EGF receptor gene

(28) and c-erb B-2 (29) as well as other members of this cell surface receptor/ tyrosine kinase family (22).

Cell surface receptors are believed to be key components of the biological control network that governs cellular growth and differentiation, and overexpression of these receptors may play a major role in the initiation and progression of certain neoplasia. In a few cases of glioblastoma and some other fresh tumors, structural alterations of the EGF receptor gene have been observed (30,31). A well known example of the correlation between the EGF receptor gene and neoplasia is erythroblastosis which is caused by an avian leukosis virus (ALV) induced structural alteration of the c-erb B gene (32). ALV integrated into the erb B locus results in overexpression of a truncated EGF receptor (33). In our study, the HBV integrated cellular erb B-like gene is expressed in the patient examined. These results are consistent with the hypothesis that the HBV genome contributes to malignancy by cis-activation of a nearby cellular gene.

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