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A t(3;8) Chromosomal Translocation Associated with Hepatitis B Virus Integration Involves the Carboxypeptidase N Locus

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Integrated hepatitis B virus (HBV) DNA is found in the great majority of human hepatocellular carcinomas, suggesting that these viral integrations may be implicated in liver oncogenesis. Besides the insertional mutagenesis characterized in a few selected cases and the contribution of viral transactivators to cell transformation to malignancy, HBV has been shown to generate gross chromosomal rearrangements potentially involved in carcinogenesis. Here, we report a t(3;8) chromosomal translocation present in a hepatocellular carcinoma developed in noncirrhotic liver tissue. One side of the translocation, in 8p23, is shown to be in the vicinity of the carboxypeptidase N gene, a locus that is heavily transcribed in liver tissue and frequently deleted in hepatocellular carcinomas and other epithelial tumors. The other side of the translocation, in 3q27-29, is widely implicated in several types of translocations occurring in different malignancies, such as large-cell lymphomas. The present data strongly support a model in which HBV-induced chromosomal rearrangements play a key role during multistep liver oncogenesis.

Chronic infection of the liver by hepatitis B virus (HBV) is the main cause of hepatocellular carcinomas (HCC) in the world and is responsible for several hundred thousand deaths each year (17). During the last decade, the finding of integrated HBV sequences in a high proportion of HCC suggested that viral insertions may be involved in tumorigenesis (for reviews, see references 19 and 34). However, the precise role of HBV integrations remains elusive. Different models have been formulated to explain HBV-associated hepatocarcinogenesis (for reviews, see references 3 and 28). The insertional-mutagenesis model implicates viral integration in the vicinity of cellular genes important for cellular growth and/or differentiation. This has been previously exemplified by the finding, in two independent tumors, of HBV integrations in the genes encoding retinoic acid receptor β and cyclin A (7, 38). Alternatively, genomic instability generated by viral integrations represents a potential pathway leading to cellular transformation. It has been shown that in early tumors, a single integration is often detected, contrasting with the multiple-integrant pattern found in late tumors (24). Previous reports have shown that such integrants could mediate chromosomal rearrangements, such as duplication, inversion, deletion, and translocation (15, 21, 35, 36, 41). Current data support the hypothesis that these genomic modifications lead to the loss of critical genes.

It has been shown that 60 to 90% of HCC, depending on the geographical area, develop on cirrhotic livers. Because cirrhosis resulting from hepatocellular necrosis and inflammation occurs as a nonspecific preneoplastic lesion (25), we were interested in investigating HBV integrations in early HCC developing on noncirrhotic livers in order to define any relevant transforming events directly linked to the viral integration process.

An HCC and the adjacent noncirrhotic liver tissue were obtained from patient 105, a hepatitis B surface antigen-positive 40-year-old woman from Shanghai, People's Republic of China. Tissue samples were submitted to protease treatment, and genomic DNA was extracted with phenol. Southern blot hybridization with a total viral genome probe of cellular DNA digested with *Hin*dIII, an enzyme which does not cleave the HBV genome, revealed that the tumor (designated 105T) contained a single HBV DNA integration. The nontumorous part of the liver (105NT) did not exhibit any unique integrated sequence or any detectable free viral DNA (Fig. 1A). However, because of the high background level of the probe, we cannot rule out the possibility that there were multiple integrations at random sites in the 105NT DNA. The 105T DNA was partially digested with *Sau*3AI and size fractionated on a sucrose gradient. Fragments from 15 to 30 kb in length were isolated, purified, and ligated into *Bam*HI arms of a λDash II vector (Stratagene). Recombinant phages were packaged in vitro and adsorbed onto *Escherichia coli* LE392 cells. About 1 million PFU was plated and screened with α-^{32}P-labeled HBV DNA. Three phage clones were isolated from this library. A restriction map of the 31-kb genomic locus covered by the three overlapping clones is shown in Fig. 2B. The structural organization of the integrated HBV DNA was determined by fine restriction mapping (Fig. 2A) and sequencing (data not shown). The sequence is similar to those of HBV clones of the *adw* subtype. The integrated DNA corresponds to a 3.6-kb rearranged fragment composed of three segments of 2, 0.4, and 1.2 kb in which the central segment is in an inverted orientation with respect to the others. Two of the four viral-DNA–viral-DNA junctions (positions 1797 and 1820) mapped in the cohesive-end region of the HBV genome, close to the first 11-bp direct repeat, similar to what was observed in other integrations (8, 15, 21, 32). The other two viral-DNA–viral-

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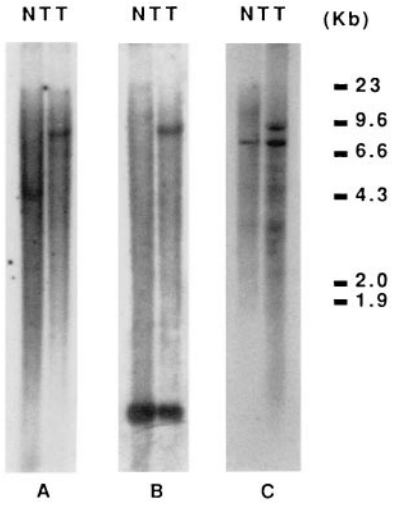

FIG. 1. Southern blot analysis of tissue samples from patient 105. Lanes NT and T represent 20 μg of *Hin*dIII-digested cellular DNA from the nontumorous and the tumorous parts of the liver, respectively. The probe used was either a complete HBV genome (A), the C3NR flanking fragment (B), or a CNH flanking fragment (C) (Fig. 2B). The DNA markers are indicated.

DNA junctions were located within the core gene (positions 2013 and 2232). The two host-DNA–viral-DNA junctions were located in the pre-S/S region (positions 3029 and 235). The complex structure of this integration suggests that it resulted from the recombination of two primary integrants (35).

As determined by Northern (RNA) blot analysis of mRNAs isolated from the tumor after phenol-chloroform extraction and affinity purification, the integration locus produced two transcripts of 2.1 and 2.8 kb containing HBV sequences (data not shown). The smaller mRNA species most probably represents the normal pre-S2/S transcript, while the larger one, whose size does not correspond to that of any known viral transcript, is likely to result from read-through transcription from viral to cellular DNA sequences.

To investigate whether HBV became integrated in the vicinity of a cellular gene in the human genome, we searched for nonrepetitive DNA sequences in the cellular DNA region encompassing the viral insertion. The detailed analysis of cellular DNA flanking sequences revealed the presence of a 460-bp fragment free of repetitive sequences (designated CPL) situated 500 bp away from the left extremity of the integrant (Fig. 2B). Hybridization of CPL to Northern blots of total and polyadenylated RNAs from normal adult livers identified an abundant 3-kb transcript (data not shown). Subsequent sequencing of the fragment followed by computer-assisted analysis revealed a remarkable homology (80% identity) with the 3′ untranslated region of the cDNA corresponding to the human carboxypeptidase N high-molecular-weight subunit (CpN), an enzyme highly expressed in liver (Fig. 3) (33). This fragment was thus named CPL, for carboxypeptidase N-like sequence. The CPL fragment also exhibited 74% identity to the human-brain-expressed sequence tag M85482 (1) (Fig. 3). Attempts to detect a transcription product by reverse transcription-coupled PCR experiments using CPL-specific oligonucleotide primers on multiple human tissue mRNAs were unsuccessful. In addi-

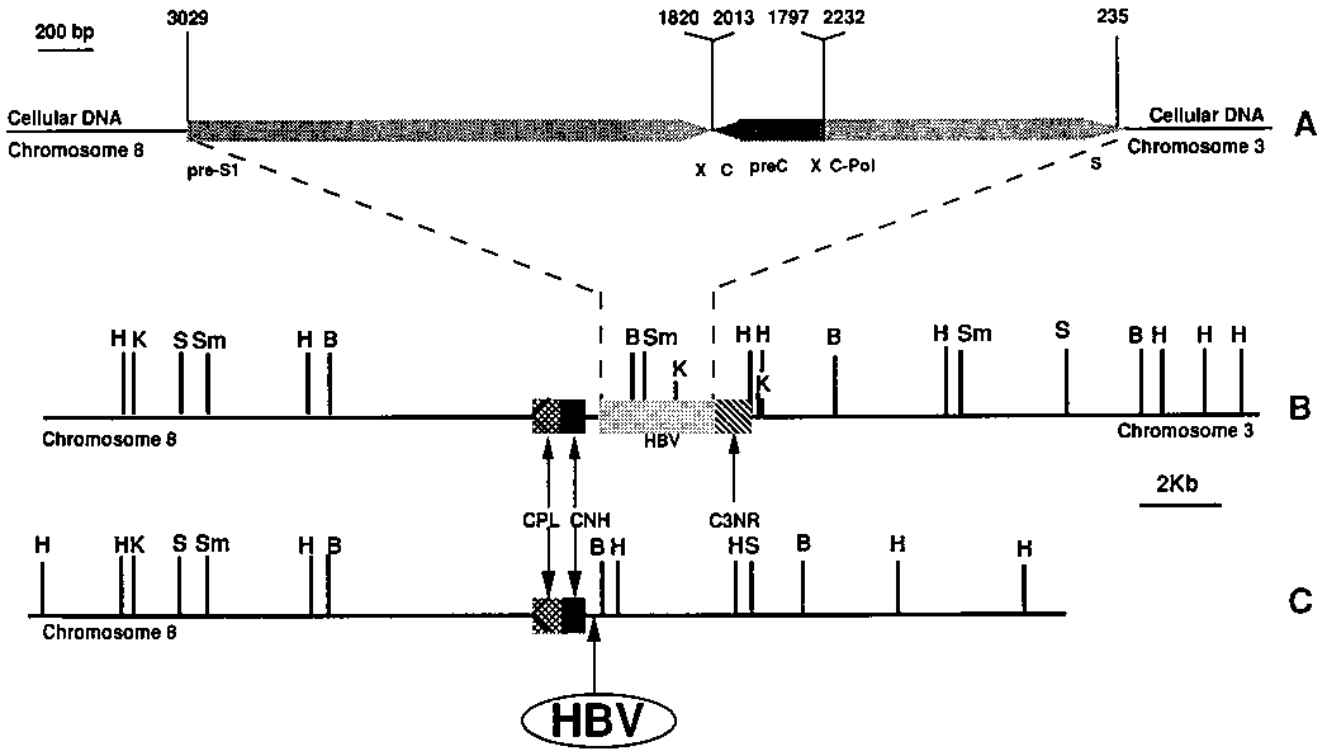

FIG. 2. (A) Organization of the 3.6-kb HBV integrant in tumor 105T. The three segments of integrated viral DNA are indicated by the contrasting stippling of the bar. Both the viral DNA open reading frames and the nucleotide numbers located at cellular-DNA–viral-DNA and viral-DNA–viral-DNA junctions are displayed. The numbering starts from the *Eco*RI site in the *adw* subtype (37). The central viral sequence is in an inverted orientation with respect to the rest of integrated viral DNA. (B) Unified restriction map of the 31-kb HBV integration region in tumor 105T, deduced from the three overlapping phage inserts. Lines represent anonymous cellular sequences. The different probes are identified. Abbreviations for restriction sites: H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; Sm, *Sma*I; B, *Bam*HI. (C) Restriction map of the 28-kb unoccupied integration locus on chromosome 8. The HBV insertion site is indicated. Restriction sites are as in panel B.

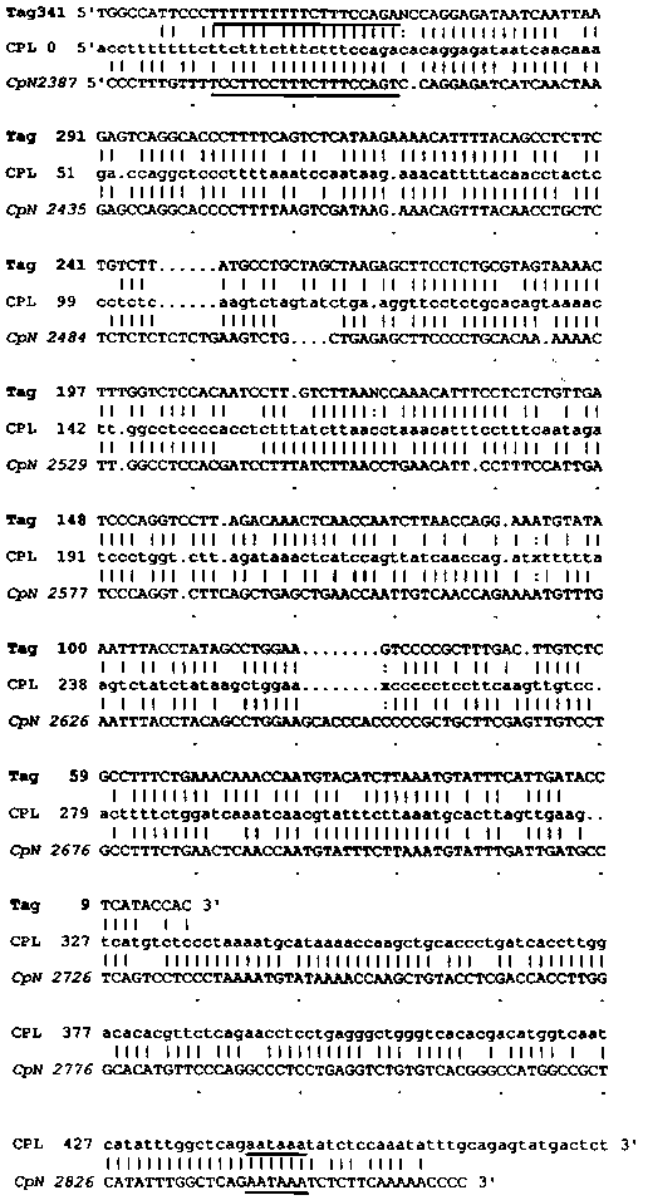

FIG. 3. Nucleotide sequence alignment of the expressed sequence tag M85482 (1), the CPL cellular DNA fragment flanking the HBV integration in tumor 105T, whose position is shown in Fig. 2B and C, and the 3′ untranslated region of the CpN cDNA (33). The pseudosplicing acceptor sites [$(C/T)_{11}$N(C/T)AG//G] (22) and the putative polyadenylation signal sites (AATAAA) are underlined. Numbering of M85482 and CpN is according to the Gen-Bank-EMBL data bank.

tion, the screening of 10^6 clones from a human liver cDNA library with the CPL probe led to the isolation of approximately 300 CpN cDNA clones but failed to reveal any clone containing the CPL sequence. Consequently, it is likely that the 3-kb transcript detected by the CPL probe resulted from a cross-hybridization with the CpN sequences.

When used as probes on a Southern blot from *Hin*dIII-digested 105T DNA, both the CNH fragment, a 420-bp sequence nonhomologous to the carboxypeptidase N sequence but contiguous to the CPL sequence (Fig. 2B), and a 700-bp single-copy flanking fragment, referred to as C3NR, adjacent to the right end of the integrant (Fig. 2B) revealed the 9.0-kb band detected by the HBV probe (Fig. 1A through C). However, these two probes failed to detect a common unrearranged allele. The C3NR fragment revealed a 1-kb band, while the CNH probe detected a 7.4-kb hybridizing band (Fig. 1B and C). This result led us to hypothesize that the HBV provirus was involved in a chromosomal rearrangement. To further characterize this putative rearrangement, we cloned the unoccupied locus from a normal human genomic library using CNH as a probe. Seven overlapping clones spanning a 28-kb region of genomic DNA were isolated. The restriction map of the HBV integration region in its germ line configuration is shown in Fig. 2C. The analysis of the unoccupied region up to 12 kb upstream of the CPL locus did not reveal any further homology with the carboxypeptidase N cDNA sequence. Interestingly, the homology breakpoint was located at the edge of an acceptor-like sequence of splice junction (Fig. 3) (22). In addition, the analysis of the unoccupied locus did not allow recovery of the C3NR fragment, suggesting that a gross chromosomal change, such as long-range deletion or translocation, occurred subsequent to HBV integration.

To address whether this was the case, we decided to assign to their chromosomal loci both the C3NR and the CNH fragments (Fig. 2B). In situ hybridization was carried out on chromosome preparations obtained from phytohemagglutinin-stimulated human lymphocytes according to the procedure described by Mattei et al. (20). The results allowed us to map the CNH and the C3NR probes to the 8p23 and the 3q27-29 regions, respectively (Fig. 4A and C). We concluded that the HBV integration in tumor 105T is associated with a sporadic t(3;8)(q27-29;p23) translocation. Concurrently, using a carboxypeptidase N high-molecular-weight-subunit cDNA as a probe and following the techniques described above (20), we mapped the carboxypeptidase N gene, whose localization in the human genome was not known, to 8p22-23, close to the HBV integration site (Fig. 4B). These data suggest that the HBV integration site on chromosome 8 is located in a nontranscribed locus that derived from duplication of the functional carboxypeptidase N high-molecular-weight-subunit gene. Such genetic organization has already been described for other hepatic genes as well as for the human β-globin gene cluster on chromosome 11, which contains two nonfunctional pseudogenes: Ψβ1 and Ψβ2 (6, 12, 31). The CpN-related sequence tag, M85482, expressed in human brain and showing a limited genetic drift, provides additional support for the hypothesis of a carboxypeptidase N gene cluster region in 8p22-23.

Chromosomal translocations in human cancers have recently been recognized as major events leading to cell transformation by activating the transcription of proto-oncogenes or creating tumor-specific fusion proteins (26). So far, molecular studies of translocations have focused primarily on hematological malignancies. Nonetheless, recurrent chromosomal aberrations have also been shown to occur in solid tumors. Specific translocations have been observed in a growing number of soft tissue tumors, such as Ewing's sarcoma, liposarcoma, and alveolar rhabdomyosarcoma (2, 5, 9), but are still considered rare events in human epithelial neoplasia (27). A recurrent t(3;8) translocation has been described for familial renal cell carcinomas (4); however, the segments involved (3p14 and 8q24.1) are different from those characterized in tumor 105T. Recently, a high rate of loss of the 8p22-23 locus has been reported for many human epithelial malignancies, including HCC (11, 13, 39). Emi et al. showed this region to be deleted in 48% of examined primary liver cancer cases (11). It is believed that the deletion of one or more unidentified tumor suppressor genes located on the short arm of chromosome 8

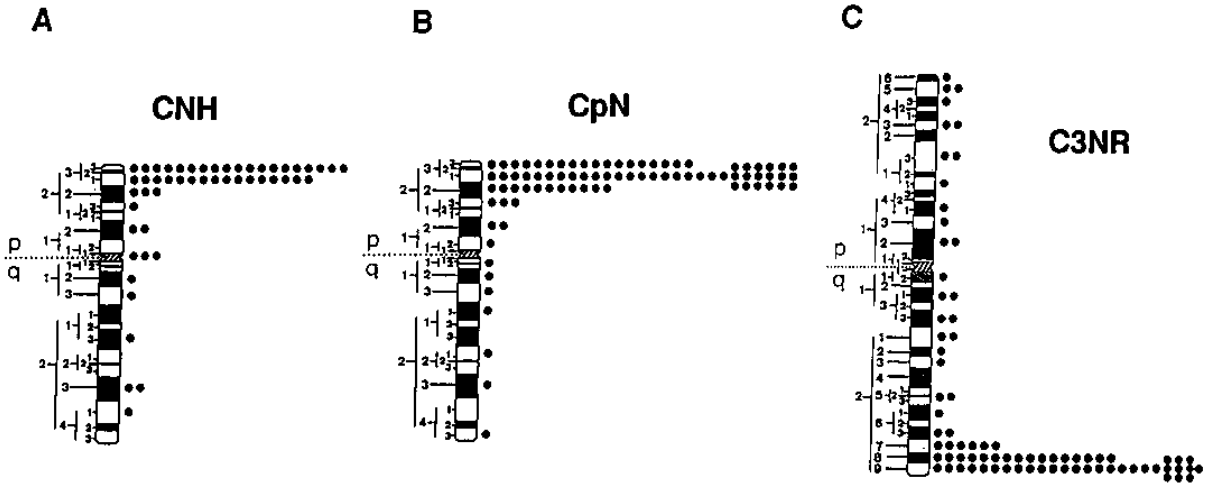

FIG. 4. Ideograms of chromosomes 8 (A and B) and 3 (C). (A) Assignment of the CNH probe corresponding to the left flanking sequence of HBV integration in tumor 105T. (B) Assignment of the CpN gene. (C) Assignment of the C3NR probe corresponding to the right flanking sequence of the HBV integrant in tumor 105T.

may be responsible for the development of various carcinomas (29). Recently, another multiple-copy region, the NAT locus, encoding a cluster of three distinct *N*-acetyltransferases has been characterized in 8p23 (14). Genetic defects in these enzymes, implicated in xenobiotic metabolism, have been associated with a susceptibility to cancer (29).

The 3q27-29 region, located at the right extremity of the HBV integration in tumor 105T, has been repeatedly implicated in chromosomal rearrangements encountered in various tumor types and has been proven to contain genes playing a major role in transformation. A t(3;12)(q27-28;q13-15) translocation was found in 15% of adipose tissue tumors, and the 3q27 locus, including the *bcl-6* proto-oncogene, is implicated in different types of translocations identified in non-Hodgkin's lymphomas (10, 16, 18, 23, 30, 40).

Four HBV-mediated chromosomal translocations, t(17;18), t(5;9), t(X;17), and t(17,7), have been described so far (13, 18, 28). Although the contributions of such sporadic translocations to liver carcinogenesis are not known, one may hypothesize that the recombination of two chromosomal loci, triggered by HBV integration, results in the activation of a proto-oncogene product or in the creation of fusion proteins. In the case of tumor 105T, it is possible that the emergence of a t(3;8) translocation-bearing HCC may result, at least partly, in a growth advantage conferred by a rearrangement that fuses a heavily liver-transcribed locus, frequently implicated in human carcinomas in 8p23, with another chromosomal region critical for cell growth control in 3q27-29. Although the exact contribution of the virus in tumor 105T is not fully understood, the observations reported here support the notion that HBV integration in selected loci of the human genome may contribute to multistep liver tumor progression. The present report suggests that this contribution could be mediated either by the fusion of two sequences, leading to inappropriate expression of cellular proto-oncogenes, or by the deletion of one copy of a tumor suppressor gene in 8p. The extensive study of the regions derived from chromosomes 3 and 8 that are involved in the t(3;8) translocation in patient 105 should provide valuable information on the specific molecular alterations associated with HBV integration in this patient. This should hopefully lead to new insights into the biology of HBV and its relevance to human liver carcinogenesis.

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