Features of Two Hepatitis B Virus (HBV) DNA Integrations Suggest Mechanisms of HBV Integration

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Two integrated hepatitis B virus (HBV) DNA molecules were cloned from two primary hepatocellular carcinomas each containing only a single integration. One integration (C3) contained a single linear segment of HBV DNA, and the other integration (C4) contained a large inverted duplication of viral DNA at the site of a chromosome translocation (O. Hino, T. B. Shows, and C. E. Rogler, *Proc. Natl. Acad. Sci. USA* 83:8338-8342, 1986). Sequence analysis of the virus-cell junctions of C3 placed the left virus-cell junction at nucleotide 1824, which is at the 5' end of the directly repeated DR1 sequence and is 6 base pairs from the 3' end of the long (L) negative strand. The right virus-cell junction was at nucleotide 1762 in a region of viral DNA (within the cohesive overlap) which shared 5-base-pair homology with cellular DNA. Sequence analysis of the normal cellular DNA across the integration site showed that 11 base pairs of cellular DNA were deleted at the site of integration. On the basis of this analysis, we suggest a mechanism for integration of the viral DNA molecule which involves strand invasion of the 3' end of the L negative strand of an open circular or linear HBV DNA molecule (at the DR1 sequence) and base pairing of the opposite end of the molecule with cellular DNA, accompanied by the deletion of 11 base pairs of cellular DNA during the double recombination event. Sequencing across the inverted duplication of HBV DNA in clone C4 located one side of the inversion at nucleotide 1820, which is 2 base pairs from the 3' end of the L negative strand. Both this sequence and the left virus-cell junction of C3 are within the 9-nucleotide terminally redundant region of the HBV L negative strand DNA. We suggest that the terminal redundancy is a preferred topoisomerase I nicking region because of both its base sequence and forked structure. Such nicking would lead to integration and rearrangement of HBV molecules within the terminal redundancy, as we have observed in both our clones.

The association of hepatocellular carcinoma (HCC) with long-term persistent hepatitis B virus (HBV) infection in humans was recognized soon after discovery of the virus (29). Prospective studies have shown that HBV carriers have a 200-fold-greater risk of HCC than uninfected individuals and that male HBV carriers have a greater than 40% lifetime risk of HCC (1). Persistent infection with the animal hepadnaviruses woodchuck hepatitis virus, ground squirrel hepatitis virus, and duck hepatitis B virus also leads to HCC in their respective hosts (16, 27, 33). The discovery that HCCs arising in woodchuck hepatitis virus and HBV carriers contain clonal hepatitis virus DNA integrations raised interest in the potential role of integrations in oncogenesis (6, 25, 28). Cloning and sequencing of integrations from woodchuck HCCs have demonstrated two instances of woodchuck hepatitis virus integration into the *c-myc* gene (13), and one cloned HBV integration occurred in a retinoic acid receptor gene (3). In another case, HBV integration was associated with the deletion of cellular DNA at chromosome 11p13 (22). This region of chromosome 11p13 is believed to contain a tumor suppressor gene, the loss of which can lead to Wilms's tumor and hepatoblastoma (14). Loss of alleles on chromosomes 11p and 13q has been shown to occur at a high frequency in HBV-associated HCCs (32a). These examples support the notion that in some instances hepadnavirus integration may play a direct role in mutagenesis of cellular controlling genes, leading to cancer.

In contrast to the above results, the vast majority of integrations cloned from HCCs are not associated with

known cellular oncogenes or a common cellular DNA sequence. The structure of integrated viral DNA varies considerably among independent HCCs. Some integrations contain viral sequences which are colinear with the viral genome, and others contain highly rearranged viral genomes (19, 20, 34). Integrations are also associated with micro- and macrodeletions and rearrangements of cellular DNA (10, 18, 22). The frequent occurrence of large deletions, inverted duplications, and translocation of cellular DNA at many HBV integration sites, in association with rearranged HBV molecules, has led to the notion that postintegration rearrangement is an important mechanism involved in generating mutations in cellular DNA. Such rearrangement may completely eliminate HBV DNA from specific cellular sites, in which case the mutagenesis mechanism could be described as a hit-and-run phenomenon. However, direct evidence for excision and reintegration of an integration has not yet been demonstrated.

The first report that primary HBV integration events might occur via a site-specific or site-preferential mechanism was the observation of two virus-cell DNA junctions which occurred within an 11-base-pair directly repeated sequence (DR1 and DR2) in the HBV genome (7). Subsequent studies have confirmed that the DR1 and DR2 sequences are highly preferred sites of HBV integration (19). The DR1 and DR2 sequences are present at the boundaries of the cohesive overlap region of viral open circular molecules and are the initiation sites for synthesis of the long (L) negative and short (S) positive strands, respectively (8, 15). The DR1 sequence is the site of a 9-base-pair terminal redundancy in the L negative strand which is generated during reverse

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transcription of viral pregenome RNA (15). Shih et al. (26) have proposed that the terminal redundancy in the L negative strand facilitates recombination with cellular DNA by strand invasion of the 3' end of the L negative strand into a recessed 5' end of cellular DNA. They have also proposed a unified approach which is based on the structure of the open circular molecule to explain the various structures of integrations. Their model categorized viral integrations into four groups according to their end specificity and strand polarity. Thus, the four groups include integrations with one virus-cell junction at DR1 with the viral DNA extending either downstream through the core gene (group I) or upstream through the X gene (group II) and integrations with one virus-cell junction at DR2 with the viral sequences extending either through the core gene (group III) or the X gene (group IV) (see Fig. 1A). Molecules which could serve as substrates for integration would include open circular molecules in replication complexes which are recycled to the nucleus (31). These molecules also accumulate in some chronically infected liver tissues in which integration occurs at a low frequency (24) and are often clonally propagated in nodular growths in precancerous liver tissue (21, 25).

Our approach to investigating the mechanism of integration events has been to clone and sequence HBV integrations and also the unoccupied cellular DNA site. The integrations described in this report have the common characteristic that they both contain a nearly complete viral genome, with one junction, either virus-cell (C3) or virus-virus (C4), in the (DR1) region of open circular HBV DNA. Our analysis of cellular integration site C3 has led to the identification of a microdeletion in cellular DNA and a region of 5-base-pair homology between cellular and HBV DNA at the junction opposite the DR1 sequence. This has allowed us to propose a specific mechanism for the integration of both ends of integration C3. We suggest that this molecule may represent a prototype integration of an open circular HBV DNA which has not experienced postintegration rearrangement. The inversion of viral sequences in the second clone (C4) at the site of a chromosome translocation further implicates the DR1 region in integration and postintegration rearrangement mechanisms possibly involving specific cellular enzymes.

MATERIALS AND METHODS

The primary HCCs (C3 and C4) in this study were obtained from two male Japanese HBV carriers at autopsy. Analysis of the genomic DNA revealed a single HBV integration in each tumor. These integrations were cloned in the *Hind*III site of lambda bacteriophage Charon 30, and the structure of the integrated HBV DNA was determined by restriction endonuclease analysis and electron microscopy as previously reported (10). The *Hind*III inserts of clones C3 and C4 were subcloned as 4.8- and 9.0-kilobase (kb) fragments in pBR327 and were designated plasmids pBC3-4.8H and pBC4-9.0H, respectively (Fig. 1). We also subcloned a 1.2-kb *Bam*HI fragment of C4 which contains the right virus-cell DNA junction and designated it pBC4-1.2B. Normal cellular DNA across the C3 integration site was cloned in the *Hind*III site of Charon 30 by ligating a 1.4- to 1.7-kb fraction of *Hind*III-digested normal C3 DNA into Charon 30 and screening the library with a flanking probe from clone C3. The cellular *Hind*III fragment was 1.6 kb and was further subcloned into the *Hind*III site of pBR327 (designated pBC3-1.6H).

The junction fragments used for sequencing integration C3 were the left 1.2-kb *Hind*III-*Bgl*II fragment and the right

0.4-kb *Bam*HI-*Bgl*II fragment from clone pBC3-4.8H, and for integration C4 the left 0.7-kb *Hind*III-*Bam*HI fragment, the internal 0.2-kb *Bgl*II fragment containing the HBV inversion, and the right 1.2-kb *Bam*HI fragment from clones pBC4-9.0H and pBC4-1.2B were used. The fragments were isolated by preparative agarose gel electrophoresis, were labeled at the 3' end with ³²P, and were isolated by strand separation in acrylamide gel electrophoresis. The purified single-stranded fragments were then sequenced by the Maxam-Gilbert method (17). The unoccupied cellular DNA corresponding to the C3 HBV integration site was determined by sequencing the 1.6-kb *Hind*III genomic clone of the unoccupied site from its right *Bgl*II site. The DNA sequence obtained was compared with published sequences of HBV DNA to determine the integration site, and the cellular sequence was analyzed for homology to known genes by using the fast N program to screen the gene bank. No significant homology with known cellular genes was detected by this analysis.

RESULTS AND DISCUSSION

In a previous report, we have described the cloning of two HBV integrations from hepatocellular carcinomas of two Japanese males (10). Restriction endonuclease analysis of the clones showed that the integrated HBV sequences in clone C3 constituted a complete, or nearly complete, HBV genome in which one virus-cell DNA junction was in the immediate vicinity of the DR1 sequence and the other virus-cell DNA junction was within the cohesive overlap region. The second integration contained two nearly complete linear HBV genomes linked together in an inverted orientation at the site of a chromosome 17:18 translocation. Since these integrations contained linear HBV sequences, they were particularly interesting to sequence because they might provide insight into the integration and rearrangement mechanisms of HBV.

Sequence analysis of the C3 HBV integration and unoccupied cellular DNA. The left virus-cell DNA junction of integration C3 was located within the 1.2-kb *Hind*III-*Bgl*II fragment, and the right virus-cell DNA junction was located within a 0.4-kb *Bam*HI-*Bgl*II fragment. The junctions were sequenced as described in Materials and Methods. The sequence of the unoccupied cellular DNA was determined by cloning a 1.6-kb *Hind*III fragment of cellular DNA from patient C3 and sequencing across the HBV integration site as described in Materials and Methods. DNA sequence analysis located the left virus-cell DNA junction at nucleotide 1824 in the viral genome (Fig. 1), which corresponds to the 5' end of the DR1 sequence and is 6 base pairs from the 3' end of the HBV L negative strand (Fig. 1). The right virus-cell DNA junction was located between nucleotides 1757 and 1762. The exact position of this junction could not be determined because the viral DNA was homologous to 5 base pairs of cellular DNA at the integration site. Sequencing the normal cellular DNA also showed that 11 base pairs of cellular DNA were deleted at the integration site. The deleted bases were immediately adjacent to the region of 5-base-pair homology with viral DNA. Strictly speaking, the deletion could have occurred before HBV integration; however, if viral DNA base paired with cellular DNA during integration, it is likely that the 11 base pairs of single-stranded cellular DNA were excised during the integration process (see below; Fig. 2). The position of the DR1 sequence at the left virus-cell junction in C3 is consistent with the strand invasion model proposed by Shih et al. (26) for

A

B

C

FIG. 2. Proposed mechanism of integration of HBV DNA forming integration C3. Base pairing of the 5' region of HBV L minus strand DNA with cellular DNA at a cellular replication fork and strand invasion by the free 3' end of the L minus strand are illustrated. The integration is formed when a cellular polymerase switches strands to the invading 3' end of HBV DNA at the DR1 sequence nucleotide (1824) and synthesis of HBV DNA is completed by either cellular or viral polymerases. Nicking and ligation of the DNA strands (*) by cellular enzymes completes the integration process and generates a microdeletion of cellular DNA. —, Viral L negative strand DNA; - - -, newly synthesized viral S plus strand and newly synthesized cellular DNA; —, cellular DNA; →, directly repeated cellular DNA sequence at the virus-cell DNA junctions. Nucleotides 1824 and 1762 are endpoints of viral DNA, and DR1 is the 11-base-pair directly repeated sequence in HBV DNA.

group 1 HBV integrations. The region of 5-base-pair homology between viral and cellular DNA at the end opposite the DR1 sequence and an accompanying microdeletion of cellular DNA allow us to propose a mechanism for this integration event (Fig. 2). According to the mechanism, the free 3' end of the HBV L negative strand invades cellular DNA, perhaps at a replication fork, and subsequent replication of the cellular DNA across the invading HBV strand, by strand switching of a cellular polymerase, would fix the virus-cell junction at the DR1 sequence. The cellular polymerase would then copy the HBV genome by displacing the S positive strand, or the polymerase could be displaced after synthesis of only the free terminally redundant segment of HBV DNA (nucleotides 1818 to 1826) and synthesis of the HBV DNA could be completed by a viral polymerase which

is present at the growing 3' end of the S positive strand. The viral polymerase is present on DNA in core particles which are believed to recycle into the nucleus (31). According to our data, the second virus-cell DNA junction was probably formed when the 5' region of the viral L negative strand base paired with its cellular DNA homolog (Fig. 2). This would have caused 11 base pairs of cellular DNA to remain single stranded and to be deleted in the C3 clone. Alternatively, it is possible that the first step of integration was the base pairing of linearized HBV DNA with cellular DNA and that this served to anchor the HBV molecule in the cellular DNA so that the free 3' end of the L negative strand could then integrate at the DR1 sequence. The nonhomologous recombination mechanism described above undoubtedly involves the participation of cellular nucleases and ligases for DNA repair and synthesis. In the next section we will discuss how one such enzyme, topoisomerase I (Topo I), may have been involved in integration or rearrangement of HBV DNA in the C4 integration.

Sequence analysis of the C4 HBV integration. Restriction endonuclease analysis of the C4 HBV integration showed that it contained two inverted copies of the HBV genome. The precise locations of the left and right virus-cell DNA junctions were determined by sequencing the 0.7-kb *HindIII-BamHI* fragment (left junction) and the 1.2-kb *BamHI* fragment (right junction). The HBV inversion site was sequenced by isolating the 0.2-kb *BglII* fragment from the center of clone C4 and sequencing it in both directions. The inverted structure of the HBV DNA resembled that of two group I integrations which had become linked in an inverted manner. Both the left and right virus-cell junctions were integrated in the cohesive overlap region at nucleotides 1798 and 1773, respectively. The HBV inversion point joined HBV molecules at nucleotides 1959 and 1820 of viral DNA (Fig. 1C). Two-base-pair homology existed between the inverted segments at the inversion, and nucleotide 1820 is the 3' end of the L negative strand of HBV open circular DNA. Inversions of this type, between different regions of HBV DNA, are very common among HBV integrations. Out of 32 integrations recently tabulated, 12 contained an inverted structure and 7 carried a sequence in the cohesive overlap region at either a virus-virus or a virus-cell DNA junction (19, 30).

Tokino et al. have proposed that there may be a cohesive-overlap-activating mechanism which stimulates integration and recombination of activated HBV DNA ends in the region of the DR sequences (30). Although there is no direct evidence for such an activating mechanism for HBV at present, it is known that integrated simian virus 40 DNA can

FIG. 1. HBV genomic map and sequencing of HBV integrations. (A) Circular map of HBV DNA illustrating the positions of the DR1 and DR2 sequences in the genome. Roman numerals I, II, III, and IV represent virus-cell DNA junctions and polarity of HBV sequences in the four categories of HBV integrations proposed by Shih et al. (26). The L minus strand (—) and the S plus strand (+) are labeled. The DNA sequence in the vicinity of the DR1 and DR2 (boxed sequences) sites, the terminally redundant region of the L negative strand, and the locations of the 5'-binding protein, the S positive strand RNA primer, and the pregenome mRNA cap site are illustrated below the map. HBV genes on the viral map are the core (C gene), surface antigen (S gene), polymerase (P gene), and X genes. (B) Linear genetic map of integrated HBV sequences above the restriction endonuclease map of the same sequences in clone C3. The DNA sequence of the left and right virus-cell DNA junctions are shown below the restriction map. The DNA sequence of normal cellular DNA at the HBV integration site along with the 11-base-pair deletion are shown on the bottom line. Sequences of HBV DNA (capitals) and cellular DNA (lowercase) are shown. The region of homology between HBV DNA and cellular DNA at the right virus-cell junction (---) and a 5-base-pair directly repeated cellular DNA sequence at the integration site (→) are indicated. (C) Inverted HBV integration designated C4 at the site of chromosome translocation t(17:18) (qter,q11). Top two lines: genetic map of inverted HBV sequences and restriction endonuclease map of the integrated HBV in clone C4. Bottom line: DNA sequence at the left and right virus-cell DNA junctions and the HBV DNA inversion point. Nucleotide 1820 of HBV at the inversion point is the 3' end of the HBV DNA minus strand. Nucleotide numbers are in reference to the unique *EcoRI* site in the HBV genome.

be induced to excise from chromosomal DNA at specific sites in somatic cell hybrids which become permissive for viral replication (5). The excision is believed to be mediated by Topo I, since Topo I nicks simian virus 40 DNA in vitro at the same sites at which excision occurs in vivo (4). Topo I can also link heterologous DNA fragments at its nicking sites (12). A study of nonhomologous recombination in parvoviruses has shown that recombination between viral and cellular DNA sequences is enhanced when the cellular DNA sequences are enriched for preferred Topo I cleavage sites and that recombination is also enhanced by the presence of a DNA sequence motif of the type 5'-CT(A/T)T(C/T)T-3' in viral DNA (11). In this context, it is interesting that the S positive strand of HBV DNA has this exact consensus sequence (5'-CTTTT-3') between nucleotides 1820 and 1825 in the highly preferred integration region. Since the 3' end of the L negative strand varied between nucleotides 1818 and 1820, the S positive strand will be unpaired in at least one position in this region. Therefore, cutting of the S positive strand by Topo I at an unpaired base between nucleotides 1800 and 1825 would generate a linear molecule which could recombine with cellular DNA at the DR1 junction. Topo I remains bound to the 3' phosphate of DNA at its nicking site, which in this case is the S positive strand. Such bound Topo I molecules have been shown to link heterologous cellular DNA fragments (12). Therefore, both of the HBV DNA ends generated by Topo I nicking might be thought of as the activated ends proposed by Tokino et al. (30). In support of this model, Topo I has been shown to cleave the S positive strand of woodchuck hepatitis virus open circular DNA at the nick in the L negative strand (i.e., its 3' end) immediately adjacent to the DR1 sequence (H. P. Wang and C. E. Rogler, manuscript in preparation).

Reports which have compared integrations in primary tumors with those in cell lines derived from the tumors have observed deletions and rearrangement of the integrations during establishment in culture (32). These postintegration rearrangements are also likely to occur during chronic infection as a consequence of continuous cellular regeneration. When the inverted HBV segment present in the C4 integration was introduced into a transgenic mouse, the sequences were unstable (9). High-molecular-weight rearranged viral genomes called novel forms have been cloned from chronically infected liver and may be the result of a rearrangement process during reverse transcription (23). In the case of yeast Ty elements, which utilize an RNA intermediate for transposition, rearrangements are common during transposition (2).

One consequence of postintegration rearrangements which is significant regarding cancer is that such rearrangements, whether they involve excision, transposition, or translocation of DNA, result in the gradual accumulation of mutations in the cellular genome. The accumulation of mutations will invariably cause a cell to become predisposed to malignant transformation in a multistage process involving the loss of function of controlling genes. Furthermore, postintegration rearrangements will result in chromosome aberrations which are no longer linked to viral DNA. This was clearly the case for the C4 translocation in which the reciprocal chromosome arm (18q11-centromere) did not contain HBV DNA (10).

The mechanisms by which integration occurs are incompletely understood. The two models of integration mentioned above, (i) recombination of viral and cellular DNA in a short region of homology and strand invasion of the opposite end and (ii) nonhomologous recombination medi-

ated by cellular enzymes, such as Topo I, are two mechanisms which are likely to generate integrated and rearranged viral molecules in infected hepatocytes. The structure of the substrate molecules for integration is probably an important determinant of the structure of the final integration. Sequencing of integrations from tissues which exhibit replication blocks may yield new insights into the integration process, and the adoption of cell culture systems to induce integration and select those cells containing new integrations is a possible approach for the future.

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