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Cloning and Structural Analysis of Integrated Woodchuck Hepatitis Virus Sequences from a Chronically Infected Liver

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We have isolated and determined the structure of a recombinant clone in lambda phage Charon 30 which contains woodchuck hepatitis virus sequences integrated in woodchuck genomic DNA sequences. This clone, in contrast to previously reported clones (Ogston et al., Cell **29**:385–394, 1982), was isolated from a chronically infected liver which never developed hepatocellular carcinoma. Southern blot analysis of viral sequences in the clone in conjunction with electron microscope heteroduplex analysis showed that the integrated viral sequences did not contain internal rearrangements, as have those from hepatomas, but were colinear with the cloned viral genome except for the deletion of approximately 500 base pairs of viral sequences (between positions 1,000 and 1,550 on the viral map). Therefore, the integration was probably a defective genome incapable of supporting viral replication. However, the complete open reading frames coding for the viral X, core, presurface, and surface antigen genes were present, indicating that the viral sequences could code for viral antigens. Southern blot analysis of the normal cellular flanking sequences, using flanking sequence probes from the clone, showed that no detectable rearrangements of cellular DNA (less than 50 base pairs) had occurred at the site of viral integration.

Woodchuck hepatitis virus (WHV) is one of four members of the newly recognized "hepadna" group of viruses (including also hepatitis B virus [HBV], ground squirrel hepatitis virus, and duck HBV) (16). The hepadna viruses have many common characteristics, including the overall structure of the virus, similar size and unusual structure of the viral DNA, similar organization of viral genes, immunological cross-reactivity, and the tendency to cause persistent infections (9, 10, 21, 23). The cytopathic effects of these viruses, however, vary greatly in their respective hosts. WHV closely resembles the human virus (HBV) in that it causes a variety of cytopathic effects including chronic persistent hepatitis and chronic active hepatitis similar to that observed in humans. Chronic active hepatitis in woodchucks has been shown to lead, almost invariably, to hepatocellular carcinoma (HCC) (19; G. Tyler, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1984).

Significant advances in our understanding of the hepadna virus life cycle have recently been made. Summers and Mason (22) have isolated immature virion cores from duck hepatocytes infected with duck HBV. The cores contain a genome-sized RNA which is reverse transcribed by an endogenus polymerase (reverse transcriptase) activity. The full-length minus-strand DNA thus generated is then used as a template for plus-strand DNA synthesis. Full-length RNA transcripts coded by the DNA minus strand have been identified in duck liver (11). These RNA molecules are likely candidates to be precursors of those packaged into cores for reverse transcription. This replication mechanism, although similar to retroviruses, is not exactly identical because a protein bound to the 5′ end of the minus strand is believed to function as a primer for reverse transcription (13) instead of a tRNA, as is used by retroviruses (7). All four of the hepadna viruses have a protein bound to the 5′ end of the virion minus strand (21).

Integrated hepatitis virus DNA has been found in almost all HCCs arising from liver tissue which has been chronically infected with either HBV or WHV. The existence of these viral integrations in hepatomas has implicated them in the oncogenic process. However, the study of cloned viral integrations (2, 8, 14) has not yet revealed a specific mechanism for oncogenesis induced by such integrations. The presence of multiple integrations in tumors, which are presumably of clonal origin, indicates that viral integrations may occur in cells before the cell becomes transformed. These integrations would be clonally propagated once a cell became transformed. Several investigators have used the Southern blot technique to demonstrate the presence of apparently integrated HBV DNA sequences in the high-molecular-weight DNA from nontumorous chronically infected liver tissue (1, 3, 18). In their experiments, hybridization was observed as either a diffuse smear in the high-molecular-weight DNA region or, in some cases, as specific bands. A diffuse smear of hybridization could be due to the random integration of HBV in different cells, and weak bands could be generated by localized clonal propagation of cells (focal clonal growths) containing such integrations.

We have used molecular cloning in an effort to directly search for integrated WHV DNA in chronically infected hepatocytes. This approach has led to the discovery of long forms of WHV DNA in chronically infected nuclei which we called "novel forms" (17). We have isolated similar novel forms of ground squirrel hepatitis virus from persistently infected ground squirrel hepatocytes, using molecular cloning (P. L. Marion et al., manuscript in preparation). We now report the structural characterization of an additional form of WHV for chronically infected hepatocytes in which WHV DNA is integrated in woodchuck cellular DNA sequences. In brief, our findings show that the integrated viral sequences could not function as a provirus; however, they

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could support the production of viral core, surface, and X antigens during persistent infection.

MATERIALS AND METHODS

Animals and strains. Woodchuck HW197 (*Marmota monax* L.) was housed at the Penrose Research Laboratory of the Zoological Society of Philadelphia, Philadelphia, Pa. This animal had chronic active hepatitis for several years before it died without ever developing a hepatoma. DNA for this study and a previous study was obtained from a liver biopsy sample taken in October 1980. The tissue sample showed no signs of HCC, and the nuclear DNA contained an abundance of covalently closed circular and open circular molecules.

Lambda phage vector Charon 30 (15) was kindly supplied by F. R. Blattner. Recombinant DNA was packaged in vitro in *Escherichia coli* LE392 by the method of Enquist and coworkers (4, 5).

Cloning and Southern blot analysis and electron microscopy. Nuclear DNA was extracted from liver HW197 as previously described (14). Lambda phage Charon 30 DNA and DNA from clone HW197-2 were isolated by the method of Vande Woude et al. (24) with minor modifications reported earlier (17). The cloning experiment in which partial *Eco*RI digests of HW197 nuclear DNA were cloned into Charon 30 was reported earlier (17). Three WHV-containing clones obtained by this procedure were reported earlier to contain novel forms of WHV. In this report, we describe the structural characterization of a fourth clone obtained by the same procedure which contains WHV sequences integrated in cellular DNA.

Southern blot analyses of the cloned DNA and woodchuck genomic DNAs were carried out as previously reported (17), as was the electron microscopy of cloned DNA and heteroduplex analysis. The filamentous phage recombinant M13-WHV was used in heteroduplexes with HW197-2. This clone contains the entire WHV genome cloned into the *Eco*RI site of vector M13mp7 and was kindly donated by W. Mason.

RESULTS

Clone HW197-2 was isolated from a library of recombinant lambda phage containing nuclear DNA from a section of liver HW197 which showed no evidence of HCC. Animal HW197, from which the liver DNA was obtained, had chronic active hepatitis with a high level of virus production and this animal died later without ever developing HCC. This is unusual for woodchucks with chronic active hepatitis in the Penrose Research Laboratory colony.

Clone HW197-2 was analyzed for viral sequences by digesting the cloned DNA with various restriction endonucleases which cut the viral genome only one time. Restriction endonuclease digests were analyzed by Southern blotting and hybridization with cloned ^{32}P-labeled WHV DNA. The initial Southern blot (data not shown) revealed that the clone contained two *Eco*RI fragments which were 2.2 and 6.3 kilobases (kb) in length, and both hybridized to WHV sequences.

To map the position of the viral sequences in the clone, electron microscope heteroduplex analysis of the cloned DNA was conducted with an M13 clone containing the entire WHV genome (3.3 kilobase pairs) inserted into M13 at the *Eco*RI site. We reasoned that hybridization of clone HW197-2 to the WHV-M13 clone would cause the M13-WHV sequences to be circularized if the internal *Eco*RI site in HW197-2 was viral DNA. A structure containing circularized WHV sequences would be easily distinguishable when analyzed by electron microscope heteroduplex analysis. A heteroduplex was constructed between three molecules, clone HW197-2, M13-WHV, and Charon 30, and a representative heteroduplex is shown in Fig. 1. Charon 30, the vector with its stuffer fragment, was included in this preparation to establish the locations of the junctions between the cloned DNA and the vector arms. Hybridization of Charon 30 to its homologous arms in clone HW197-2 clearly delineated the ends of the cellular sequences in clone HW197-2 (see Fig. 1, a and a'). The unhybridized stuffer fragment of Charon 30 is visible in Fig. 1, b and b'. The heteroduplex observed clearly demonstrated that the WHV sequences present in the WHV-M13 clone were circularized when they hybridized to clone HW197-2 (see Fig. 1, c, e, and e'). The broken arms of the M13 vector are visible in Fig. 1, d, and the heteroduplex region between the WHV sequences in clone HW197-2 and M13-WHV are visible in Fig. 1, e and e'. This proved that the internal *Eco*RI site of clone HW197-2 was viral, and measurement of the length of the heteroduplex regions on either side of the internal *Eco*RI site revealed that the total length of viral sequences in the clone was 2.75 kb. A single-stranded region of 550 base pairs (bp) was present in the circularized WHV sequences of the WHV-M13 clone (Fig. 1, c). This represented the viral sequences which were missing in clone HW197-2 (approximately 550 bp between positions 1,000 and 1,550 on the viral map) (6). The lengths of the left- and right-hand cellular flanking sequences in clone HW197-2 (Fig. 1, g and f, respectively) were determined to be 5.5 and 0.6 kb, respectively, by measuring the single-stranded regions between the end of the integrated WHV sequences and the junction with the Charon 30 vector arms. All of the heteroduplexes observed, including broken molecules, were consistent with the structure presented in the intact molecule shown in Fig. 1. No inverted duplications, which would have been visualized as hairpins under the electron microscope, were observed.

Further Southern blot analysis of clone HW197-2 DNA was used as an independent method to confirm the structure of the viral sequences and to map additional restriction endonulease sites in the flanking cellular sequences. The Southern blot is presented in Fig. 2, and the final restriction endonuclease map is shown in Fig. 3A along with a map of the complete WHV genome (Fig. 3B) and the location of the open reading frames for the viral surface antigen (S), core antigen (C), X gene (X), and pre-S sequences (preS). The measurements of viral and cellular sequences from heteroduplex analyses are presented in Fig. 3C, and a summary diagram illustrating how the observed restriction endonuclease fragments confirmed the final map is given in Fig. 3D. Figure 2, lanes 5a and 5b, shows that double digestion with *Eco*RI and *Cfo*I produced three fragments which hybridize to WHV (6.3, 1.7 and 0.6 kb). The published sequence for WHV shows a *Cfo*I site at 1,766 bp from the *Eco*RI site. Since the 0.6-kb fragment hybridized weakly to WHV, we conclude that the right-hand virus-cell junction is a short distance beyond the viral *Cfo*I site located 1,766 bp to the right of the internal viral *Eco*RI site in the clone. These results were consistent with the electron microscope heteroduplex analysis which positioned the virus-cell junction 1,750 bp to the right of the internal *Eco*RI site.

The left-hand virus-cell junction occurs very close to the 3' end of the surface antigen gene according to the electron microscope heteroduplex analysis. Southern blot analysis (Fig. 2) indicated that the *Sac*I and *Bgl*II sites located 1.2 and 1.1 kb (respectively) to the left of the internal *Eco*RI site were probably in cellular sequences because no hybridiza-

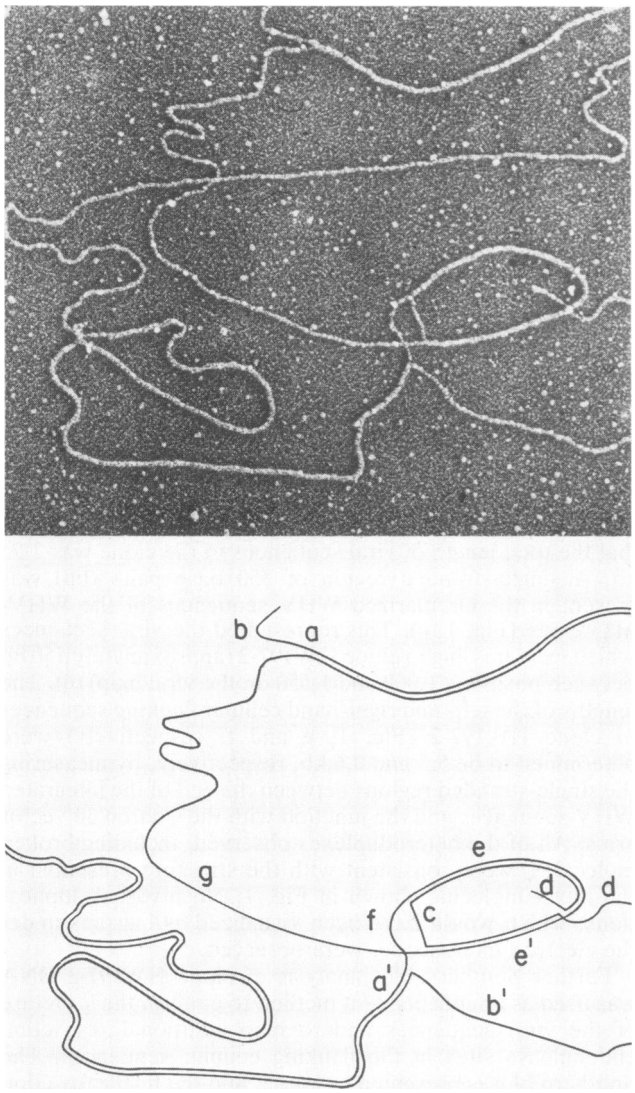

FIG. 1. Heteroduplex analysis of WHV and cellular sequences in clone HW197-2. DNA molecules used were Charon 30 (a, a,', b, b'), clone HW197-2 (whole length of heteroduplex), and M13-WHV (c, d, e, e'). Interpretation: (a and a') heteroduplex regions between vector arms of Charon 30 and clone HW197-2; (b and b') single-stranded stuffer fragment of Charon 30 vector; (c) single-stranded portion of WHV sequences in M13-WHV clone; (d) broken M13 vector arms from M13-WHV; (e and e') heteroduplex regions between WHV sequences in HW197-2 and M13-WHV clone; (f) single-stranded right-hand woodchuck cellular sequence in HW197-2; (g) single-stranded left-hand woodchuck cellular sequence in HW197-2.

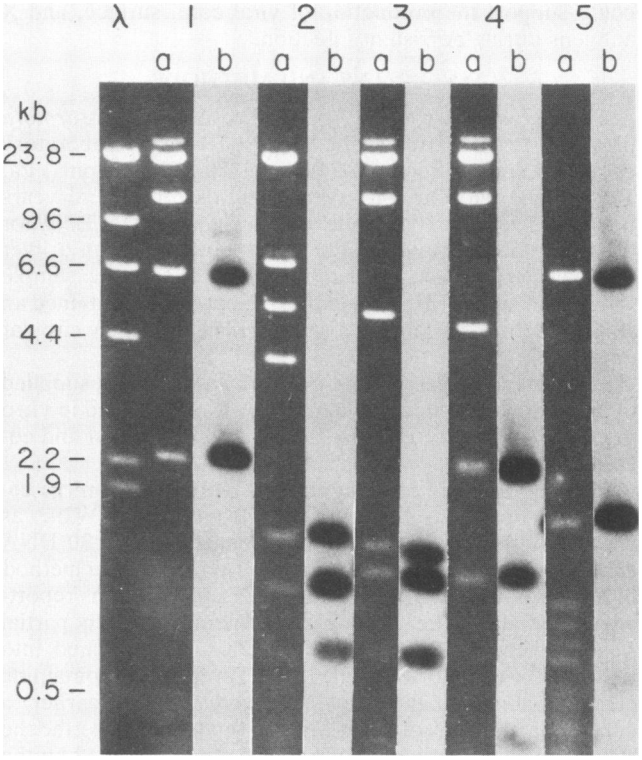

FIG. 2. Restriction endonuclease mapping and Southern blot analysis of the cellular and WHV sequences in clone HW197-2. Restriction endonuclease digests: (1a) *Eco*RI; (2a) *Eco*RI + *Bgl*II; (3a) *Eco*RI + *Sac*I; (4a) *Eco*RI + *Xba*I; (5a) *Eco*RI + *Cfo*I. (Lanes 1b to 5b) Southern blots of the corresponding lanes, hybridized with ^{32}P-labeled WHV probe. A total of 10×10^6 cpm of ^{32}P-labeled WHV DNA was prepared and hybridized as previously reported (17). Hybridization was for 45 min at 37°C. Vector DNA fragments in lanes 1a, 3a, and 4a are 33.5-, 21.5-, and 12-kb bands. Charon 30 vector fragments for lane 2a are the 21.0-, 7.0-, 4.0-, and 0.7-kb bands. Faint hybridization of the ^{32}P-labeled WHV probe to a 0.6-kb fragment in lane 5b was stronger on longer exposure of the autoradiogram. The vector fragments for the *Eco*RI-*Cfo*I digest were not mapped. Lambda, *Hin*dIII digest of wild-type lambda phage; fragment sizes designated on the left.

tion of WHV was observed to the 5.1-kb *Eco*RI-*Sac*I or the 5.2-kb *Eco*RI-*Bgl*II fragments (Fig. 2, lanes 2a and b and 3a and b). These conclusions were independently confirmed in the next experiment.

When DNA from clone HW197-2 was used to probe a Southern blot of *Eco*RI-digested, uninfected, woodchuck genomic DNA, hybridization to a single genomic fragment of approximately 6.0 kb was observed. The size of this genomic fragment was approximately equal to the length of the genomic sequences in clone HW197-2 according to electron microscope analysis. This observation raised the possibility that the integration of WHV could have occurred in cellular sequence without any detectable rearrangement (deletion or duplication) of cellular sequences. In this case, insertion of 2.75 kb of viral DNA would have resulted in the generation of a new fragment of 8.75 kb which was approximately the size of the cloned DNA fragment.

It was possible to test this hypothesis in the following manner. Restriction endonuclease mapping had established the presence of single *Bam*HI, *Bgl*II, and *Sac*I sites in the left-hand flanking cellular sequences of clone HW 197-2. If no major rearrangements of cellular sequences had occurred during viral integration, then the cellular *Bam*HI, *Bgl*II, and *Sac*I restriction sites should be 4.2, 0.75, and 0.85 kb, respectively, away from the right-hand *Eco*RI site in normal uninfected woodchuck genomic DNA. A Southern blot of uninfected woodchuck DNA digested with *Eco*RI and *Bam*HI, *Sac*I, or *Bgl*II was probed with the right-hand flanking sequences of clone HW197-2 (i.e., the right-hand *Eco*RI fragment of clone HW197-2 was used). The results (Fig. 4A) show that this probe hybridized to fragments of the predicted sizes for all three double digests (4.2 kb for *Eco*RI-

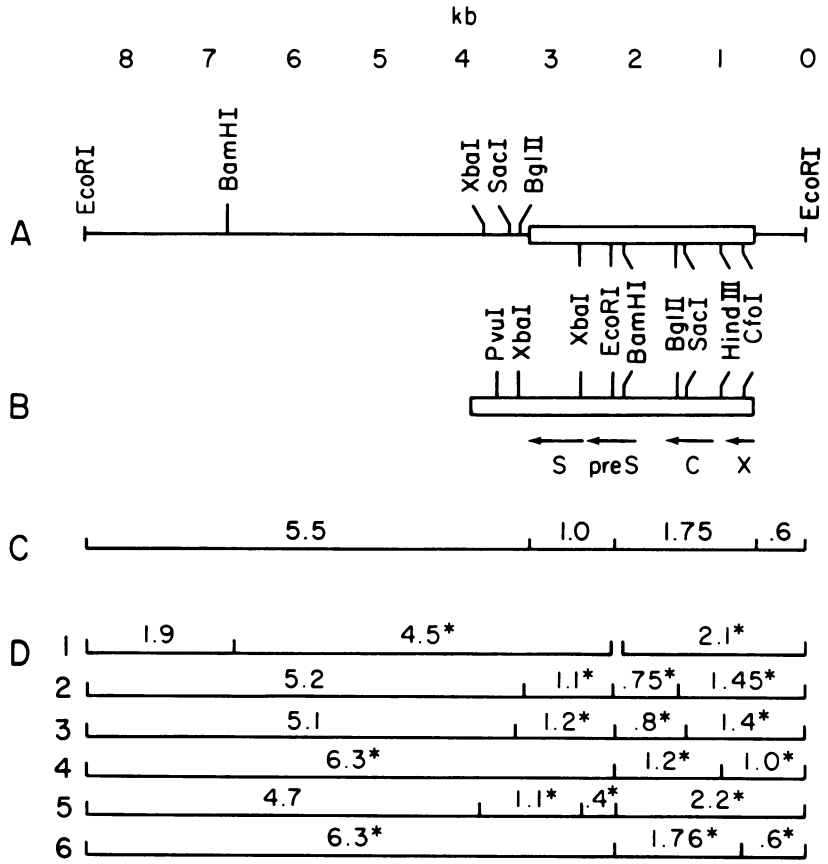

FIG. 3. Restriction endonuclease map of clone HW197-2 (A) and the complete WHV genome (B). Symbols for (A): (——) woodchuck cellular sequences; (□) WHV sequences. (B) Arrows indicate the location and direction of transcription of open reading frames corresponding to the viral surface antigen gene (S), core antigen gene (C), X gene (X), and pre-S sequences (preS). (C) Measurements of WHV and cellular sequences obtained from electron microscope heteroduplex analysis. (D) Schematic diagram of restriction endonuclease fragments produced by double digestions of HW197-2 DNA with *Eco*RI plus (1) *Bam*HI, (2) *Bgl*II, (3) *Sac*I, (4) *Hin*dIII, (5) *Xba*I, and (6) *Cfo*I. * denotes fragments which hybridize to ^{32}P-labeled WHV probe.

*Bam*HI, 0.75 kb for *Bgl*II-*Eco*RI, 0.85 kb for *Sac*I-*Eco*RI). A probe containing the left-hand flanking sequences (the left-hand *Eco*RI fragment of HW197-2) was hybridized to a second Southern blot of similarly digested woodchuck DNA. Again the probe hybridized to fragments of the sizes predicted from the restriction map of clone HW197-2 (Fig. 4B). It hybridized to *Eco*RI-*Bgl*II and *Eco*RI-*Sac*I fragments of 5.2 and 5.1 kb, respectively, and to two *Eco*RI-*Bam*HI fragments of 2.0 and 4.2 kb. The left-hand probe contains a mildly repeated sequence, and therefore a smear of hybridization was observed on Southern blots. However, intense hybridization to specific genomic bands was clearly evident and distinguishable above the background smear. These results lead us to the conclusion that, within the level of resolution of the techniques used (which should be 50 to 200 bp), there were no detectable rearrangements of cellular sequences at the site of integration of WHV DNA. Restriction maps illustrating the site of integration of viral DNA in the cellular sequences, along with a schematic summary of the Southern blot data supporting the maps, are presented in Fig. 4C and D. It is possible that short rearrangements (possibly direct duplications) of cellular sequences occurred during integration and would not be detected by the methods used. This question can be resolved by sequencing the virus-cell junctions and the normal cell sequences at the site of integration. It is very clear, however, that large rearrangements of cellular sequences did not occur at the site of viral integration as has been observed for the cellular sequences flanking WHV and HBV integrations from several HCCs (manuscript in preparation).

DISCUSSION

Persistent infections with WHV share characteristics in common with other persistent virus infections, including a minimum cytopathic effect, limited immunopathological damage to persistently infected cells, and association of virus infections with tumors (12). In an attempt to gain an understanding of the molecular basis of viral persistence in woodchuck hepatocytes, we began to study the forms of viral DNA in the nuclei of hepatocytes. The forms of DNA described in a previous report (17) include (i) covalently closed circular DNA which is the most abundant form, being present in approximately 50 copies per nucleus in liver HW197; (ii) open circular DNA (possibly generated by nicking of covalently closed circular DNA during isolation of nuclear DNA); and (iii) novel forms of WHV. These forms are composed of long stretches of viral DNA (greater than two genomes) which contain multiple rearrangements of viral sequences. These were found to be present in approximately one copy per cell in liver HW197 but may be present in higher copy numbers in other liver tissues. They may have existed as large covalently closed circular molecules which

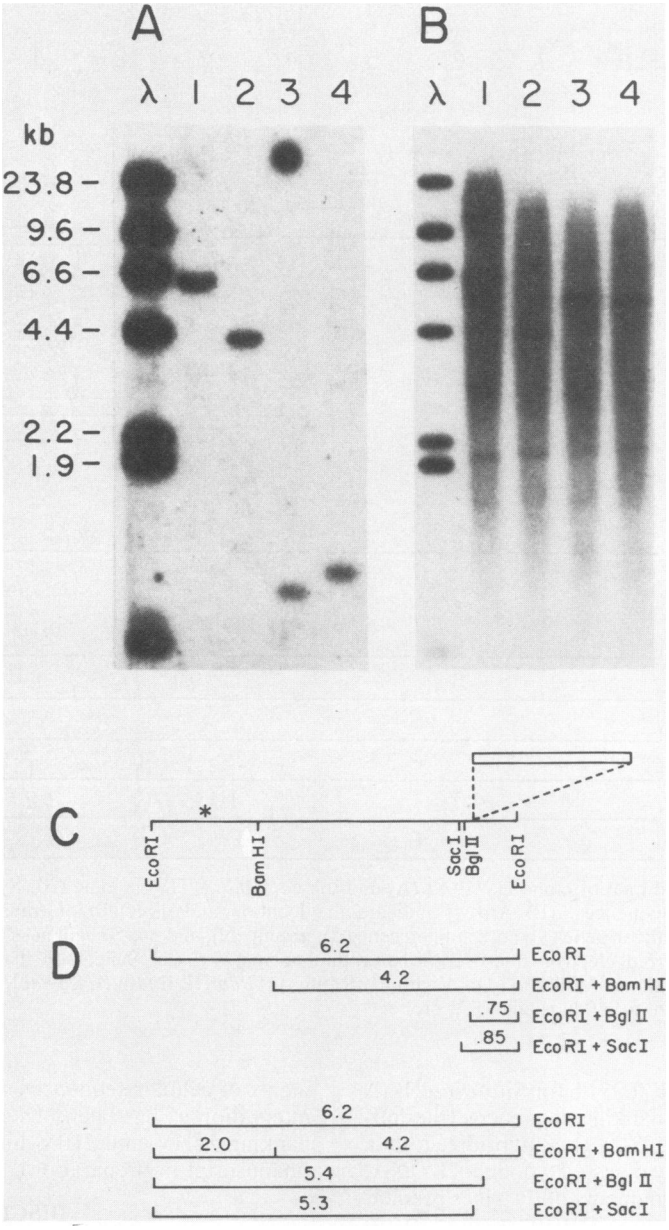

FIG. 4. Southern blot analysis of uninfected woodchuck cellular DNA digested with various restriction endonucleases and hybridized with right- or left-hand flanking sequence probes from clone HW197-2. (A) Southern blot of woodchuck DNA hybridized with the right-hand *Eco*RI fragment of HW197-2. (B) Same as in (A) except the probe was the left-hand *Eco*RI fragment of HW197-2. Restriction endonuclease digestions in (A) and (B) were: (1) *Eco*RI only; (2) *Eco*RI + *Bam*HI; (3) *Eco*RI + *Bgl*II; (4) *Eco*RI + *Sac*I. (C) Proposed site of integration of WHV DNA in woodchuck cellular sequences: (——) cellular DNA; (▭) WHV DNA. (D) Schematic diagram of results obtained from Southern blots (A) and (B) (above): restriction fragments hybridizing to the right-hand probe (upper set) and left-hand probe (lower set). * Location of repetitious sequence in left-hand flanking sequences.

were linearized by cleavage with *Eco*RI, or they may have been excised from longer stretches of rearranged viral DNA which was integrated in cellular DNA. In this report, we describe the structural characterization of a fourth form of WHV DNA which is integrated in cellular DNA in about one copy per three cells.

Direct evidence for the function of any of the above molecules in the viral life cycle or in persistent infection is completely lacking. At best, we can speculate as to their possible functions. Since the WHV life cycle may involve an RNA intermediate, similar to duck HBV, we have also conducted experiments to attempt to isolate proviruses from acutely infected woodchuck and duck livers. These experiments, conducted under the same conditions in which integrated clones were isolated, have failed to identify a clone containing a complete provirus. Therefore, the nuclear covalently closed circular DNA present in high copy number in hepatocytes is the most likely candidate to serve as the template for the RNA pregenome which would be necessary for reverse transcription.

The structural features of the integrated WHV sequences in this paper show that this integrated molecule most proba-

bly could not have supported viral replication because it contained a defective genome with a 550-bp deletion. On the other hand, the coding sequences for the pre-S, surface, core, and X genes are present in the integrated molecule, indicating that transcription of these sequences could result in production of those viral antigens. Whether this integration significantly contributed to antigen production in the infected hepatocyte in which it resided is unknown. Another intriguing observation is that the right-hand virus-cell junction of the clone is immediately adjacent to the proposed 5′ end of the viral plus strand. Previous work (2. 8, 14) has shown that virus-cell junctions in hepatomas frequently occur in the vicinity of the cohesive end region and often in the region of the genome which is single stranded in the virion DNA.

Some general characteristics of WHV integrations present in HCCs include: (i) integration at different sites in both the viral and cellular sequences in independent hepatomas; (ii) variation in the amount of viral DNA in individual integrations from less than a genome length to greater than genome lengths; (iii) complex rearrangements of viral sequences in the integrations, including inverted duplications and deletions; and (iv) rearrangements of cellular sequences at the site of viral integration (manuscript in preparation). The integration described in this paper differs from those found in hepatomas in that the viral sequences did not contain any inverted duplications and there was no detectable rearrangement of cellular sequences at the site of integration. Isolation and structural characterization of additional integrations from chronically infected tissues are in progress to determine whether these differences can be generalized.

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