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Characterization of Viral Genomes in the Liver and Serum of Chimpanzee Long-term Hepatitis B Virus Carriers: A Possible Role for Supercoiled HBV-DNA in Persistent HBV Infection

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In chimpanzee hepatitis B virus (HBV) carriers, the molecular mechanism for viral persistence has been examined by analyzing the properties of viral DNA molecules in liver and serum. Two extrachromosomal HBV-DNA molecules migrating on Southern blots at 4.0 kb and 2.3 kb were observed in chimpanzee liver DNA. There was no evidence for integration of HBV sequences into the host genome. The HBV-DNA molecule which migrated at 4.0 kb position represents a full-length "nicked," relaxed circular form, and the DNA molecules migrating at 2.3 kb position represents a supercoiled form of the HBV genome. Evidence for supercoiled HBV-DNA in serum was obtained by production of the relaxed circular intermediate upon digestion of Dane particle DNA with specific nucleases S1 and Bal 31. A possible role of these two extrachromosomal HBV-DNA molecules in the biology of hepatitis B virus infection and the mechanism for viral persistence are discussed.

Key words: hepatitis B virus, persistent viral infection, HBV-DNA, chimpanzee HBV carriers, molecular hybridization, supercoiled HBV-DNA

With the development of tests for HBsAg in the 1960s, it was found that some chimpanzees were carriers of this antigen. Acute viral hepatitis could be induced in these primates by injection of serum from human hepatitis B virus (HBV) carriers [1, 2], but the histologic lesions in chimpanzees were usually milder than in man [3,4]. Recently, however, two types of experimental hepatitis B have been described in chimpanzees, one with rapid resolution of acute hepatic inflammation and the other with smoldering features of continued portal inflammation histologically defined as chronic persistent hepatitis [5]. Some animals also become long-term HBV carriers maintaining elevated serum levels of HBsAg, antibody to hepatitis B core antigen (anti-HBc), HBeAg, and Dane particles [5, 6]. The Dane particle in serum (42 nm in

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diameter) has morphologic and other properties to suggest that it represents the complete virion of HBV [7]. Its known structural components include a nucleocapsid core of 27 nm containing virion DNA and two proteins referred to as core antigen (HBcAg) and "e" antigen (HBeAg), a unique virion DNA polymerase, and an outer coat containing viral surface antigen (HBsAg). The viral genome is a small circular DNA molecule that is partially double-stranded [7, 8]. The long strand, a, is of constant length (~3,200 bases) with a "nick" at a unique site, and the short strand, b, varies in length between 1,700 and 2,800 bases in different molecules. The DNA polymerase activity in the virion [9, 10] can repair the single-strand region to make fully double-stranded molecules of approximately 3,200 bp. Recent evidence indicates that a protein is covalently linked to the 5′terminus of the complete "a" strand at the nick site, but the origin and function of this protein are not yet known [11]. A protein kinase activity in the nucleocapsid particle has also been described [12].

In chimpanzee HBV carriers, the mechanism of viral persistence has been examined by analyzing the properties of viral DNA molecules in liver and serum. We have shown that in addition to heterogeneous Dane particle DNA, the liver of chimpanzees contains HBV-DNA in a covalently closed, supercoiled circular configuration. We have also reported evidence for a small percentage of supercoiled HBV-DNA genomes within the serum Dane particle population [13]. From these studies it was hypothesized that most of the Dane particles may represent incomplete viral forms (those containing partially double-stranded DNA circles), and that those molecules that contain supercoiled HBV-DNA may represent the complete infectious form of hepatitis B virus. The incomplete forms of HBV might be acting as defective interfering (DI) particles and may play a significant role in the establishment and maintenance of persistent HBV infection.

RESULTS

Nonintegrated State of HBV-DNA Molecules in Chimpanzee Liver

Using a hot phenol-SDS extraction procedure, we previously reported that DNA isolated from the liver of five chimpanzee carriers contains HBV-DNA molecules that do not appear to be integrated into the host genome [6]. When DNA was isolated by hot phenol-SDS extraction from the liver of one of these chimpanzees (CH30), we detected two discrete HBV bands in a DBM–paper blot hybridized with purified, cloned (^{32}P)HBV-DNA (Fig. 1, lane A). The electrophoretic migration position of the more slowly migrating HBV band is 4.0 kb compared to linear double-stranded DNA standards; the faster migrating band is located at 2.3 kb position (Fig. 1, lane A). No hybridization was observed at the migration position of the full-length, linear HBV genome (~3.25 kb).

The size and discreteness of the hybridization bands with undigested CH30 DNA (apparent migration at 4.0 kb and 2.3 kb, respectively) suggested that these molecules were extrachromosomal. Since a 3.25-kb linear full-length, HBV-DNA molecule was not detected, the nature of these other HBV-DNA bands was investigated. There was no change in the hybridization pattern when CH30-DNA was digested with Hind III, which recognizes no internal cleavage site within the HBV genome [14–16]; compare undigested DNA (Fig. 1, lane A) with Hind III digested DNA (Fig. 1, lane B). This raised the possibility that either these molecules represent linear forms of HBV, one being a subgenomic fragment at 2.3 kb and the other

containing extra DNA sequences at 4.0 kb, or that both molecules represent physical isomers of the HBV genome—eg, a circular form migrating at 4.0 kb and a supercoiled form migrating at 2.3 kb. Digestion of CH30-DNA with EcoR1, which recognizes a single site in the HBV genome [15–18], produced a new band migrating at 3.25 kb (Fig. 1, lane C) with the concomitant disappearance of the 2.3 kb band.

In order to demonstrate further the substrate-product relationship during the EcoR1 conversion of the 2.3 kb apparent sequence length molecule to 3.25 kb, a fixed amount of CH30-DNA was digested with progressively increasing amounts of EcoR1. With increasing ratios of EcoR1/DNA, hybridization at 2.3 kb decreased and eventually disappeared and a new band appeared at 3.25 kb (Fig. 2). At the highest Enz/DNA digestion ratio used (Fig. 2, lane D), it is evident that the 2.3-kb supercoiled molecule was fully converted to full-length linear 3.25 kb HBV-DNA. For reasons that are unclear, some 4.0-kb HBV-DNA remained after digestion with high amounts of EcoR1.

These results confirmed that neither the 4.0-kb nor the 2.3-kb apparent sequence length bands is integrated into host DNA, and suggests that they might represent physical isomers of the HBV genome. Both molecules hybridize to the full complement of the HBV genome as determined by hybridization to a series of purified subgenomic fragments of HBV-DNA ranging in length from 350 to 900 base pairs, taken from all regions of the genome [13]. Since all the subgenomic fragments of HBV-DNA hybridized to the "2.3-kb" component, no major deletion was present in this molecule. Its rapid migration, therefore, suggests that it might represent a supercoiled form of HBV-DNA. In the case of the "4.0-kb" molecule, it still might represent a linear molecule containing extra sequences in addition to 3.25-kb HBV-DNA or a relaxed circular form of the HBV genome.

Additional Evidence for the Circular and Superhelical Nature of the "4.0-kb" and "2.3-kb" HBV-DNA Molecules

An alternative approach to establish the supercoiled nature of the "2.3-kb" molecule was to use endonuclease S1 from Aspergillus oryzae [19]. It has recently been shown that S1 nuclease introduces highly selective cleavages into supercoiled covalently closed circular DNA [20]. The cleavage sites are small inverted repeats, separated by a nonrepetitious sequence of 2–6 base pairs which may adopt hairpin or related structures. S1 nuclease converts supercoiled DNA into a linear molecule via a two-stage reaction by introducing a "nick" in one strand at a hairpin or loop site producing an intermediate relaxed circle, followed by slower conversion to linear molecules by cleavage opposite to the "nick" [20]. At various times after S1 digestion, CH30-DNA was subjected to DBM–paper blot hybridization and the position of "2.3-kb" HBV-DNA or its digestion product was determined (Fig. 3). With 200 units of S1 nuclease per 200 μg of CH30-DNA (Fig. 3A), there was accumulation of "4.0-kb" intermediated molecules at 2′,4′,and 10′,representing the first stage in S1 nuclease digestion. Small amounts of linear HBV-DNA (3.25 kb) also appeared. At later times (30′and 60′)the proportion of "4.0-kb" HBV-DNA molecules diminished, and the amount of the linear form (3.25 kb) increased. With 400 units of S1 nuclease per 200 μg CH30-DNA (Fig. 3B), the same sequence of events occurred, but the reaction proceeded more rapidly. As S1 digestion continued toward completion (60′), the majority of supercoiled "2.3-kb" HBV-DNA was converted to 3.25-kb linear forms. Physical evidence for the supercoiled nature of "2.3-kb" HBV-DNA has also

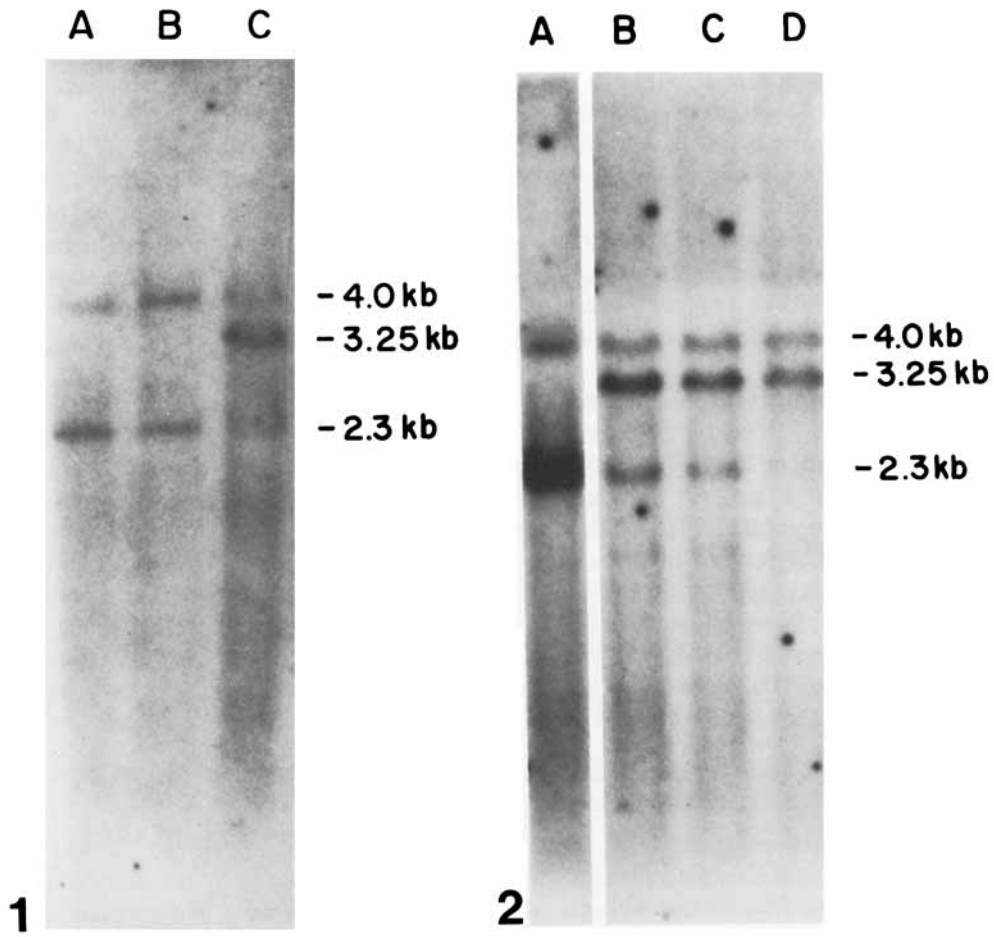

Fig. 1. Hybridization analysis of chimpanzee liver DNA after restriction endonuclease digestion. Aliquots (20 μg each) of total CH30 liver DNA were digested with a 3-fold excess of EcoR1 and Hind III restriction endonucleases. Samples were subjected to electrophoresis through a 0.8% agarose gel and then transferred to DBM paper. The paper was hybridized with (^{32}P)-labeled cloned HBV-DNA probe, washed, dried, and autoradiographed. A) Undigested CH30 liver DNA; B) Hind III digested CH30 liver DNA; C) EcoR1 digested CH30 liver DNA. Apparent molecular weights in kilobases are shown on the right.

Fig. 2. Effect of increasing enzyme: DNA ratio on chimpanzee liver DNA digestion with EcoR1. Aliquots (20 μg each) of total CH30 liver DNA were digested with increasing units of restriction endonucleases EcoR1, separately, for a fixed length of time (12 hr at 37°C for each digestion). Samples were subjected to electrophoresis through a 0.8% agarose slab gel, transferred to DBM paper, hybridized with (^{32}P)-labeled cloned HBV-DNA probe, washed, dried, and autoradiographed. A) Unrestricted CH30 liver DNA; B) CH30 liver DNA digested with EcoR1 at a concentration of 0.1 units per μg DNA; C) CH30 liver DNA digested with EcoR1 at a concentration of 0.5 units per μg DNA; D) CH30 liver DNA digested with EcoR1 at a concentration of 3 units per μg DNA. Apparent molecular weights in kilobases are shown on the right.

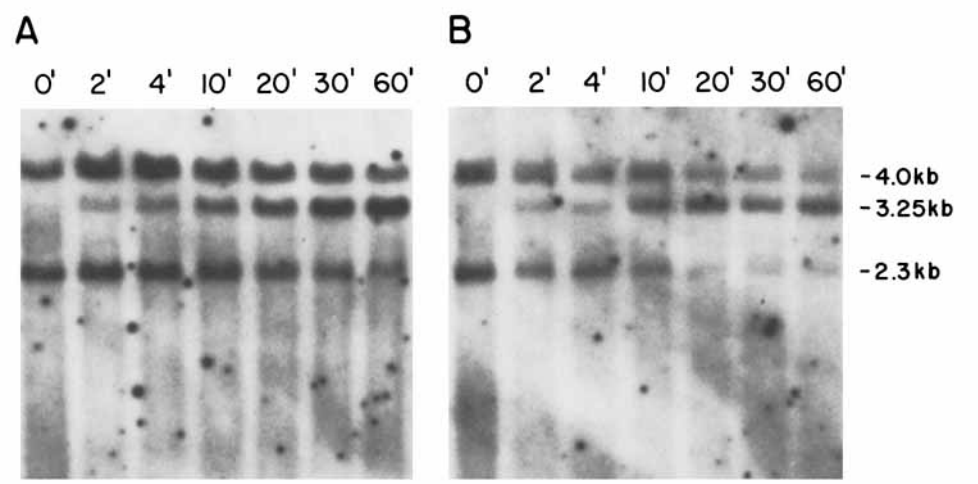

Fig. 3. Kinetics of digestion of CH30 liver DNA with S1 nuclease. Panel A shows the kinetics of digestion of total CH30 liver DNA (200 μg) with 200 units of S1 nuclease. Panel B shows the same experiment with 400 units of S1 nuclease. Equal aliquots ($\sim$25 μg of DNA) were taken from the reaction mixture at various times during digestion from 0'to 60'as indicated on the top of the autoradiogram. S1 nuclease digestion was stopped by the addition of SDS, NaEDTA, and the material was phenol-extracted followed by ethanol precipitation. After completion of the experiment, the aliquots were loaded onto a 0.8% agarose slab gel, electrophoresed, transferred to DBM paper, and hybridized with (^{32}P)-labeled cloned HBV-DNA probe. The paper was washed, dried, and autoradiographed. Apparent molecular weights in kilobases are shown on the right.

been obtained by ethidium-bromide CsCl gradient centrifugation of CH30 liver DNA [13].

Restriction endonuclease analysis of S1 cleaved chimpanzee DNA proved that the S1 cleavage site of supercoiled HBV-DNA is unique and is very close to Bam HI site lying adjacent to the EcoRl site of HBV-DNA [13]. Computer analysis of the HBV-DNA sequence within 160 nucleotides on both sides of the EcoRl site of two independent HBV-DNA clones [21, 22] shows a potential hairpin loop structure involving the EcoRl site (Fig. 4). Such a hairpin loop might exist in supercoiled HBV-DNA and serve as the recognition site for S1 nuclease.

To demonstrate the circular nature of the "4.0-kb" intermediate, we used nuclease BAL 31. Alteromonas espejiana BAL 31 nuclease is a multifunctional enzyme that contains highly specific single-stranded endonuclease activity similar to S1, and a processive exonuclease activity that simultaneously degrades both 5'and 3' termini of duplex DNA [23–25]. Figure 5 shows the position of HBV-DNA sequences at various times during digestion of CH30-DNA with BAL 31. With progression of time (5', 10', and 20'), "2.3-kb" supercoiled HBV-DNA was converted to full-size linear HBV-DNA (3.25 kb). As noted previously with S1 nuclease, a "4.0-kb" presumptive "relaxed" circular intermediate also appeared early during BAL 31 digestion (5'and 10'). At later times (30'and 60'), 3.25-kb linear HBV-DNA was progressively degraded to shorter molecules by BAL 31 exonuclease activity. It should be noted that the size of the "4.0-kb" presumptive intermediate remained unchanged throughout the entire period of BAL 31 digestion. This strongly suggests

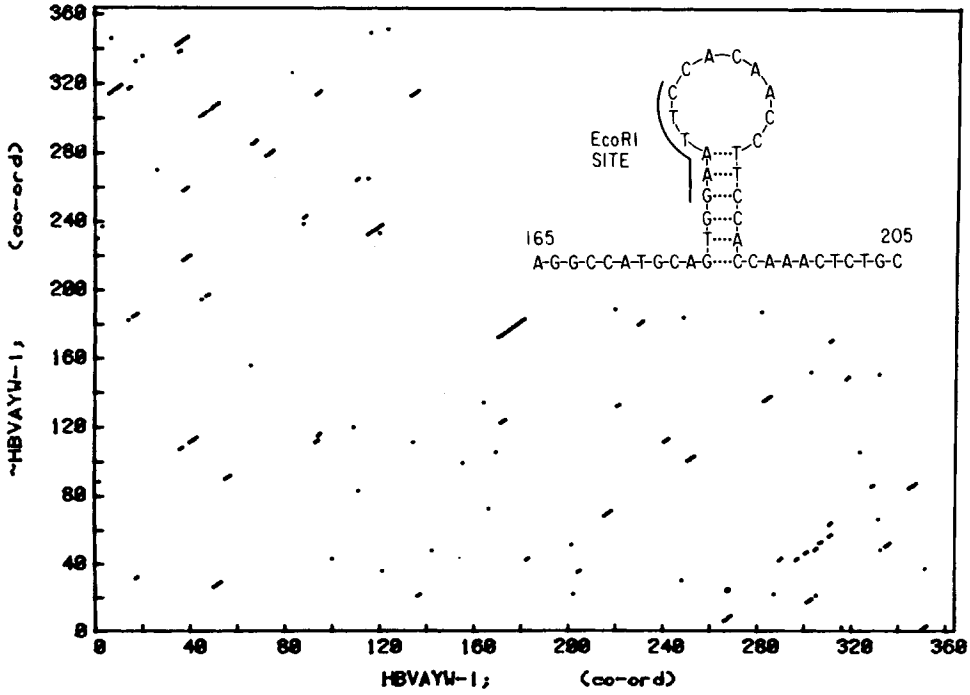

Fig. 4. Identification of a possible hairpin-loop structure of HBV-DNA within the EcoR1 site. The sequence of HBV-DNA as reported by Galibert et al [21] was analyzed within 160 nucleotides to each side of the EcoR1 site for possible regions of intramolecular base pairing into hairpin-loop structures utilizing a computer program. Regions of base pairing are represented by dots in this two-dimensional plot, and the largest such region passes directly through the EcoR1 site. A schematic diagram illustrating the sequence and configuration of the proposed hairpin loop is shown in the inset. The sequence of HBV-DNA reported by Valenzuela et al [22] provides an almost identical hairpin loop in this same region.

that the "4.0-kb" HBV-DNA molecule does not have free 5′ and 3′ ends, and probably represents a nicked "relaxed form" circle of HBV. Since supercoiled HBV-DNA was linearized to distinct 3.25-kb molecules and no other smaller bands were produced, this suggests that supercoiled HBV-DNA was cleaved in a unique fashion by both S1 and BAL 31 nucleases.

Evidence for Supercoiled HBV-DNA in Human and Chimpanzee Serum Dane Particles

The above results demonstrated that supercoiled HBV-DNA exists in persistently infected CH30 liver. We wished to determine whether this supercoiled HBV molecule was also present in serum Dane particles. To ensure quantitative isolation of HBV-DNA, Dane particle concentrates from chimpanzee or human serum were treated with proteinase K and the nucleic acid was subsequently phenol-extracted. When this DNA was electrophoresed, blotted, and hybridized with cloned (^{32}P)HBV-DNA, diffuse hybridization was observed between 3.25 kb and 2.0 kb (Fig. 6A). If

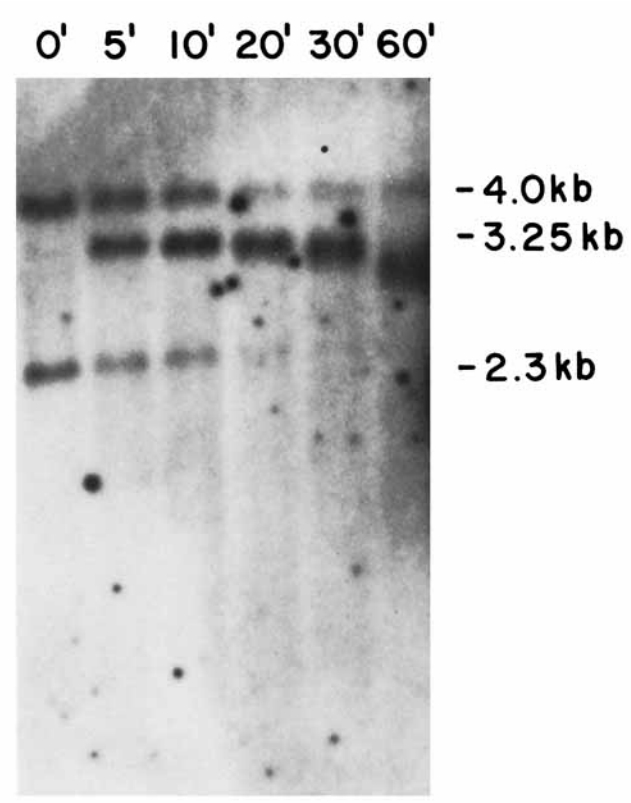

Fig. 5. Kinetics of digestion of chimpanzee liver DNA with BAL 31 nuclease. About 125 μg of total CH30 liver DNA was digested with 30 units of BAL 31 nuclease. Equal aliquots (~20 μg of DNA) were taken from the reaction mixture at various times during the digestion from 0'to 60'as indicated on the top of the autoradiogram. BAL 31 nuclease digestion was stopped by the addition of SDS, NaEDTA, NaEGTA, and the aliquots were heated at 65°C for 10'.Nucleic acid was phenol-extracted followed by ethanol precipitation. After completion of the experiment, the aliquots were loaded onto a 0.8% agarose slab gel, electrophoresed, transferred to DBM paper, and hybridized with (^{32}P) -labeled cloned HBV-DNA probe. The paper was washed, dried, and auto radiographed. Apparent molecular weights in kilobases are shown on the right.

"2.3-kb" supercoiled HBV-DNA were present in Dane particle DNA, detection would be difficult within this heterogeneous mass. Therefore, a different approach was utilized to identify supercoiled HBV-DNA in serum Dane particle DNA. We took advantage of the fact that S1 and BAL 31 nuclease linearize supercoiled DNA in a two-stage reaction (see Figs. 3, 5). If supercoiled HBV-DNA molecules were present within Dane particle DNA, we should be able to generate the "4.0-kb" intermediate by gentle digestion with S1 or BAL 31. Figure 6B shows a "4.0-kb" HBV-DNA band in CH30 serum Dane particle DNA within 1–2 min after addition of nuclease S1. This band is not present in either chimpanzee or human Dane particle DNA not digested with S1 (Fig. 6A). With longer digestion time, "4.0-kb" molecules disappeared and were apparently converted to 3.25-kb linear forms that were not

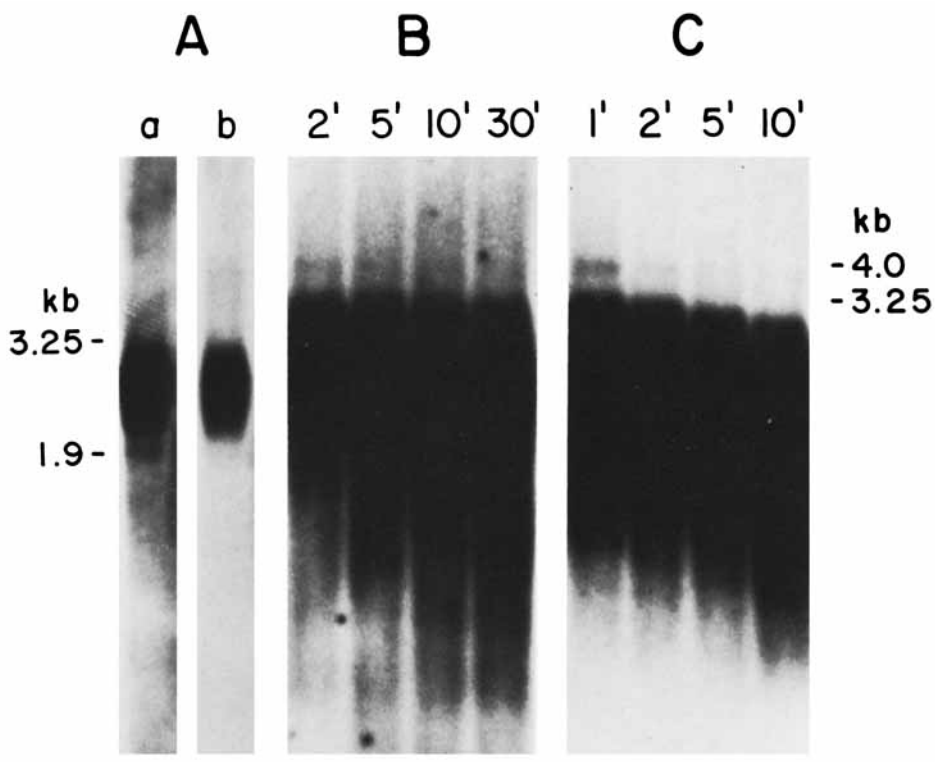

Fig. 6. Kinetics of digestion of concentrated Dane particle DNA from chimpanzee and human serum with S1 or BAL 31 nuclease, respectively. Panel A shows the autoradiogram of concentrated Dane particle DNA hybridized with (^{32}P)-labeled cloned HBV-DNA probe. DNA was isolated from 200 μl of a concentrated Dane particle preparation. Five to ten percent of the purified Dane particle DNA was loaded onto a 0.8% agarose slab gel, electrophoresed, and transferred to DBM paper. The paper was hybridized with (^{32}P)-labeled probe, washed, dried, and autoradiographed. Panel A, lane a: human Dane particle DNA; lane b: chimpanzee Dane particle DNA. Apparent molecular weights are shown in kilobases on the left. Panel B shows the kinetics of S1 nuclease digestion of CH30 Dane particle DNA. Panel C shows the kinetics of BAL 31 nuclease digestion of human Dane particle DNA. Purified DNA was suspended in 100 μl of 10 mM Tris HCl, pH 7.5, 1 mM EDTA. Half of this DNA solution from chimpanzee or human serum was digested with 100 units of S1 or 10 units of BAL 31 nuclease, respectively. Equal aliquots were taken from the reaction mixture at various times as indicated on the top of the autoradiogram (panel B: 2′to 30′and panel C: 1′to 10′). Nuclease digestion was stopped and the material was loaded onto a 0.8% agarose slab gel, electrophoresed, and transferred to DBM paper. The paper was hybridized with (^{32}P)-labeled cloned HBV-DNA probe, washed, dried, and autoradiographed. Apparent molecular weights are shown on the right.

detectable within the heterogeneous hybridization of Dane particle DNA. BAL 31 treatment of human Dane particle DNA also generated a "4.0-kb" band (Fig. 6C). Furthermore, there was degradation of linear 3.25 kb molecules (at 5′and 10′)by the 5′–3′ exonuclease activity of BAL 31. Some "4.0-kb" molecules remained, indicating the relaxed circular nature of this DNA. Because the single-stranded region of Dane particle DNA is heterogeneous, digestion with S1 or BAL 31 produced other lower MW HBV-DNA fragments spread diffusely throughout the gel (Fig. 6B, C).

DISCUSSION

Possible Implications of the Supercoiled HBV-DNA Molecule in Persistent HBV Infection

In this and other studies [6, 13], we have shown that persistence of HBV in the liver of chimpanzees can occur under circumstances in which we have not been able to identify by direct Southern blots the integration of viral genetic information into the host genome. It has recently been shown that bovine papilloma virus can persist as nonintegrated viral DNA in bovine cells transformed in vitro [26]. There are also reports of nonintegrated papilloma viral DNA in tumor cells derived from natural infection with papilloma virus [27–29]. Therefore, mechanisms other than integration of viral DNA may be involved in the establishment and maintenance of HBV persistence. One such mechanism might involve the production and accumulation of defective interfering (DI) particles, which have received considerable attention in the last decade [30, 31].

DI animal viruses are noninfectious viruses that interfere with the replication of standard viruses [31]. Usually, DI viruses are produced after repeated undiluted passage of the virus [31], and over 99% of the infectious virus population can be replaced by DI viruses [31]. Interference caused by "DI-like" particles has now been discovered for almost every animal virus group [31]. Although DI particles have not been thoroughly characterized in each system, there is little doubt that DI-like particles are virtually universal and can play a significant role in virus multiplication. In general, DI particles have a number of common properties—namely, inability to propagate in the absence of a helper virus (defectiveness), ability to be complemented by helper virus and to multiply in the presence of helper, ability to decrease the yield of the wild-type virus (interference), and ability to increase their proportion in the yield from cells coinfected with wild-type virus (enrichment) [31]. They are antigenically indistinguishable from their parent standard virus, and their genomes have been found to be variable in length involving deletions, substitutions, and/or duplications of small regions [31]. Finally, in long-term carrier cultures or persistently infected animals, the ratio of DI particles to standard virus often reaches $\sim 10^3$ [32–34].

In the case of DNA viruses, DI particles have been shown in persistently infected tissue cultures of papova viruses [35–38] and herpes viruses [39–41]. Extensive investigation of both SV40 and polyoma DI particles has shown that four defects occur during their generation: deletion of viral information, duplication of the origin of DNA replication in the remaining molecules, insertion of host cell DNA into the closed circular viral DNA, and polymerization of small monomer units into multimers about the same size as the standard viral genome [31]. In this light, it was particularly interesting to discover that HBV can exist in the liver of chimpanzees as closed supercoiled HBV-DNA. This raises two possibilities: First, supercoiled HBV-DNA may be a replicative intermediate in the pathway for generation of Dane particle DNA which is partially double-stranded. Second, it might have a more significant role in the biology of the virus—namely, representing the complete viral DNA structure associated with infectious particles.

The preponderant genome structure found in Dane particles (the partially double-stranded DNA molecule) is unique to the HBV family. Studies of cloned Dane particle DNAs indicate a significant heterogeneity in the HBV-DNA sequence. These differences are reflected as variations in restriction endonuclease maps indicating small deletions or substitutions [42]. For example, the single EcoRI cleavage site in

HBV-DNA is absent in certain HBV genomes [43]. Siddiqui et al [17] have shown occasional differences in restriction enzyme patterns within the same subtype of HBV-DNA isolated from different patients (eg, adW_2 and ayW_3). Among the three cloned HBV-DNAs from which the sequence has been published, two are presumed to cover the entire genome but vary in length from 3,182 to 3,221 nucleotides [42]. Sequences of six and 33 nucleotides at positions 2,354 and 2,845 in the HBV/ad clone of Valenzuela et al [22] are deleted in the clone of Galibert et al [21], and it is unlikely that these differences can be explained by artifacts generated during cloning of HBV-DNA.

It should be noted that Dane particles for most HBV-DNA studies have been obtained from patients with long-term persistent HBV infection. Therefore, variability in the DNA sequence observed in cloned HBV-DNAs might be a reflection of their defective interfering nature. In this context, it is important to mention that in terms of the amount of DNA sequence variation required to produce interference, even a single base change leading to a single amino acid substitution (as for example in a *ts* viral mutant) may be sufficient [44–48]. Finally, synthesis of a large excess of viral envelopes, accumulating as spherical and filamentous protein aggregates in infected serum [7], is another feature of persistent HBV infection consistent with interfering virus production.

The unusual structure of Dane particle DNA may also be relevant to these considerations. The most striking features are its partial double-stranded nature with a single-strand region extending anywhere from 15% to 50% of the circle length in different molecules [8, 49], the presence of a DNA polymerase activity, and the recent finding of a protein covalently bound to the 5′-terminus of the complete Dane DNA strand [11]. By analogy with bacteriophage systems [50], it has been proposed that the 5′covalently linked protein might be a viral gene product acting as a strand- and site-specific endonuclease for initiation of viral DNA replication [11]. Therefore, it is possible that the partially double-stranded structure containing a covalently linked protein might represent an incomplete replicative intermediate of the HBV genome. This intermediate might then be packaged into virions prematurely during abnormal viral morphogenesis. During normal HBV morphogenesis, the complete DNA structural form—namely, supercoiled molecules—might be associated with the infectious particle.

The observation that the infected hepatocyte synthesizes a large excess of viral envelopes [7], accumulating as spherical and filamentous protein aggregates in serum, could be explained as a result of imbalance in the temporal regulation of the viral gene expression during morphogenesis. This imbalance in the expression of viral genes during morphogenesis might also trigger an early premature packaging of protein-bound, incomplete DNA replicative intermediates.

As we have mentioned previously, one possible mechanism to account for viral persistence might involve the production and accumulation of DI particles throughout the infection. If such a mechanism operates during HBV persistence, we might expect to find a population of viral particles in the serum of long-term carriers in which the majority will represent DI particles ($>99\%$), and a very small fraction ($<1\%$) will represent standard viral particles [31, 34]. It should be emphasized that the level of closed supercoiled HBV-DNA structures in serum Dane particles represents a very low percentage ($<1\%$) as compared to that of incomplete double-stranded Dane DNA molecules ($>99\%$) (see Fig. 6B, C). Therefore, our finding of a very low level

of closed supercoiled structures in serum Dane particle DNA is consistent with this notion.

The hypothesis presented above does not exclude other mechanisms, such as abnormalities in the host cellular immune response or HBV-DNA integration, from contributing to HBV persistence under appropriate circumstances. Although the principles underlying this discussion are based on DNA structural studies, we believe that the concept of interference or generation of an excess of imcomplete or mutant viruses incapable of productive infection might be important in considering various experimental strategies to isolate the infectious agent of HBV in tissue culture. If successful, this could lead to a new understanding of the mechanism for HBV replication and its relationship to the state of persistent viral infection.

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