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The Covalently Closed Duplex Form of the Hepadnavirus Genome Exists In Situ as a Heterogeneous Population of Viral Minichromosomes

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Replication of hepadnaviruses requires a persistent population of covalently closed circular (CCC) DNA molecules in the nucleus of the infected cell. It is widely accepted that the vital role of this molecule is to be the sole DNA template for the synthesis by RNA polymerase II of all viral transcripts throughout the infection process. Since the transcriptional activity of eukaryotic nuclear DNA is considered to be determined in part by its specific organization as chromatin, the nucleoprotein disposition of the hepadnavirus CCC DNA was investigated. These studies were undertaken on the duck hepatitis B virus (DHBV) CCC DNA present in the liver cell nuclei of DHBV-infected ducks. The organization and protein associations of the DHBV CCC DNA in situ were inferred from sedimentation, micrococcal nuclease digestion, and DNA superhelicity analyses. These three lines of investigation demonstrate that the DHBV CCC DNA is stably associated with proteins in the nuclei of infected liver cells. Moreover, they provide compelling evidence that the viral nucleoprotein complex is indeed a minichromosome composed of classical nucleosomes but in arrays that are atypical for chromatin. When the DHBV chromatin is digested with micrococcal nuclease, a ladder of viral DNA fragments that exhibits a 150-bp repeat is produced. This profile for the viral chromatin is obtained from the same nuclei in which the duck chromatin shows the standard 200-bp ladder. The superhelicity of the DHBV CCC DNA ranges from 0 to 20 negative supertwists per molecule, with all possible 21 topoisomers present in each DNA preparation. The 21 topoisomers of DHBV CCC DNA are inferred to derive from an identically diverse array of viral minichromosomes. In the DHBV minichromosomes composed of 20 nucleosomes, 96.7% of the viral DNA is calculated to be compacted into these chromatin subunits spaced on average by 5 bp of linker DNA; other minichromosomes contain fewer nucleosomes and proportionately more linker DNA. Two major subpopulations of DHBV minichromosomes are detected with comparable prevalence. The two groups correspond to minichromosomes which contain essentially a full or half complement of nucleosomes. The functional significance of this minichromosome diversity is unknown but is suggestive of transcriptional regulation of the viral DNA template.

The hepadnavirus family is a small group of liver-tropic animal viruses whose virions contain an open-circular (OC) DNA molecule of 3.0 to 3.4 kb. These viruses possess a uniquely frugal genomic organization and a distinctive strategy for replication, in which the hepadnavirus genome is amplified in the synthesis of multiple copies of a terminally redundant replicative RNA termed the pregenome. Transcription of the 1.1 genome-sized pregenome RNA occurs in the nucleus of infected cells from a covalently closed circular (CCC) form of viral DNA (16, 22, 36). The pregenome RNA is encapsidated into cytoplasmic nucleocapsids within which each molecule of pregenome RNA is converted (without amplification) by reverse transcription into a specific OC duplex DNA (28). Cytoplasmic nucleocapsids then exit the cell upon envelopment at the endoplasmic reticulum and secretion via the Golgi pathway as virions. The viral CCC DNA is formed from the OC DNA present in nucleocapsids upon their entry into the cell nucleus, either (i) from parental virions originating from outside the cell or (ii) from the pool of progeny nucleocapsids formed in the cytoplasm (32). These two pathways culminate in the formation of a regulated steady-state population of 20 to 50 CCC DNA molecules per infected cell (17, 29). The half-life of these molecules in explanted hepatocyte cultures is estimated to be 3 to 5 days (3); however, their half-life in vivo remains undetermined. If they are not as stable as the nuclear DNA of the host, they must be appropriately renewed by de novo synthesis. The CCC molecules are not replicated by a semiconservative scheme but, rather, are produced only by the conservative reverse transcription pathway (32). A continued productive hepadnavirus infection, as occurs in both acute infections and many chronic carrier patients, clearly requires a persistent population of transcriptionally active CCC DNA molecules as both the source of pregenome RNA for replication and the template for mRNA synthesis and the subsequent production of all viral proteins.

The concept of a viral minichromosome was formulated to describe the organization of simian virus 40 (SV40) genomes in the nuclei of infected permissive cells (8). The viral DNA-protein complex displayed a nuclease digestion profile very

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similar to that for the bulk host chromatin. Such digestion produced the now classical 200-bp repeat "ladder" of DNA fragments characteristic of eukaryotic nuclear DNA and reflective of its association with histones into nucleosomes (13, 18). A similar organization has been found for the genomes of other viruses which can be present in cells as unintegrated nucleoplasmids. These include parental adenovirus DNA during lytic infection (30), Epstein-Barr virus DNA in nonproducer cell lines (24), herpes simplex virus DNA in latently infected brain stem (4), and cauliflower mosaic virus in infected turnip leaves (20). By contrast, however, no comparable evidence was found for a nucleosome arrangement of herpes simplex virus DNA in productively infected cells in vitro (14). Thus, it seems likely, but not inevitable, that nucleoplasmid DNA will be found organized as nucleosomes much like the host chromatin.

The pivotal role that viral CCC DNA plays in the hepadnavirus infection process prompted us to determine whether this nucleoplasmid is arranged in situ, like SV40 DNA, with a nucleosomal structure. In this paper we provide evidence that the CCC DNA molecules of the duck hepatitis B virus (DHBV)—an avian hepadnavirus—indeed exist as viral minichromosomes in the liver cell nuclei of infected ducks.

MATERIALS AND METHODS

Ducks and viruses. The DHBV-infected livers were obtained from congenitally infected ducks aged 2 to 10 weeks. Pekin and Pekin-Aylesbury cross-bred ducks were the hosts. Two independently isolated strains of DHBV were used in these experiments. They were the p2.3 strain (GenBank no. M60677), originating from an American commercial flock, and an Australian isolate from a commercial flock in Victoria (5). The p2.3 strain shows 99% DNA sequence identity with DHBV-16 (15). The full DNA sequence for the Australian strain remains undetermined; partial sequence data indicate a high (>98%) degree of identity with DHBV-16. DHBV p2.3 was clonally derived (26) from an infectious recombinant plasmid containing a tandem head-to-tail dimer of the viral DNA sequence inserted at the *Eco*RI site of pBR322; the recombinant pBR322-p2.3 plasmid was obtained from Jesse Summers. The experiments described in this report have all been performed with both virus strains, except for the experiment in Fig. 3, which was done with the p2.3 virus only.

Isolation of nuclei from duck liver. (i) Method 1. The liver was rinsed in cold isotonic solution H (0.25 M sucrose, 3 mM $MgCl_2$, 10 mM NaH_2PO_4 [pH 6.5]) and minced with scissors into 3 volumes (wt/vol) of the same solution. The liver was disrupted in a loose-fitting Dounce homogenizer, and the homogenate was strained through four layers of cheesecloth and centrifuged for 10 min at 2,000 rpm in a Sorvall SS34 rotor to pellet the nuclei. The supernatant was carefully removed, and the pellet was suspended in 7 to 10 volumes (wt of liver/vol) of 2.3 M sucrose–3 mM $MgCl_2$–10 mM NaH_2PO_4 (pH 6.5). The suspension was centrifuged for 1 h at 22,000 rpm in a Beckman type 30 rotor at 4°C; the supernatant was decanted, and the nuclei were washed twice in solution H containing 1% Triton X-100.

(ii) Method 2. The liver was removed and immediately perfused with phosphate-buffered saline (PBS). The liver was cut into small pieces and dispersed by blending in solution H containing 0.2 mM phenylmethylsulfonyl fluoride. This mixture was forced through a wire mesh, layered over Percoll, and centrifuged at 20,000 rpm in a Beckman JA-20 rotor at room temperature; the supernatant was removed by aspiration. The pellet of hepatocytes was suspended in solution H and homogenized by 10 strokes in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 2,500 rpm in a Beckman JA-14 rotor for 20 min at 4°C; the pellet was resuspended in 2.3 M sucrose–0.2 mM phenylmethylsulfonyl fluoride–3 mM $MgCl_2$–10 mM NaH_2PO_4 (pH 6.5) and centrifuged at 22,000 rpm in a Beckman SW28 rotor for 1 h at 4°C to pellet the nuclei. The whitish layer of nuclei was resuspended in solution H containing 1% Triton X-100, homogenized in a loose-fitting Dounce homogenizer, and repelleted at 2,000 rpm for 10 min at 4°C. The last two steps were repeated until the pellet appeared clean by microscopic inspection. Nuclei were counted at this stage by staining an aliquot with ethidium bromide.

The yield of cell nuclei by both methods, as calculated from the recovery of duck DNA, was variable and ranged from 60 to 90%. However, the yield of DHBV CCC DNA obtained from the isolated nuclei (per gram of duck DNA) was identical to that for whole liver and corresponded to 20 to 30 molecules per diploid cell.

Isolation of DHBV nucleoprotein complexes in sucrose gradients. Nuclei prepared by method 1 were resuspended in buffer (10 mM EDTA, 10 mM Tris-HCl [pH 7.9]), and NaCl was added to 0.2 M. After 15 min at room temperature, the lysed nuclei were centrifuged at 2,500 rpm in a Sorvall HB-4 rotor for 10 min at 4°C to pellet most of the cellular chromatin (7); the supernatant contained the DHBV nucleoprotein complex. Linear 5 to 20% (wt/vol) sucrose gradients were prepared in TSE (0.2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH 7.9]). Gradients were prepared in SW40 tubes and underlaid with a 1-ml cushion of CsCl (density, 1.4 g/ml). Samples (0.4 ml) were gently layered onto the sucrose gradients, centrifuged in a Beckman SW40 rotor for 2 h at 30,000 rpm at 25°C, and recovered in (approximately 0.4-ml) fractions collected by bottom puncture. Sedimentation rates were calibrated with SV40 form I DNA (Gibco-BRL) and [^{3}H]rRNA markers prepared as follows. Vero cell monolayers were grown to confluence in minimal essential medium plus 5% calf serum. Cultures were labeled with 0.05 mCi of [^{3}H]uridine per ml in the same medium for 16 h and extracted by a modification of the Hirt protocol (11). The cells were washed three times in PBS and lysed in 0.6% sodium dodecyl sulfate (SDS)–10 mM EDTA–10 mM Tris-HCl (pH 7.5) for 20 min at room temperature. The lysate was incubated with proteinase K (100 μg/ml) for 1 h at 37°C, made 1.0 M for NaCl, and kept for 8 h at 4°C. The mixture was centrifuged at 17,000 rpm in a Sorvall SS34 rotor for 30 min at 4°C, and the supernatant was decanted, extracted with phenol-chloroform, and mixed with 3 volumes of ethanol. The precipitated RNA was collected by centrifugation and dissolved in buffer (1 mM EDTA, 10 mM Tris-HCl [pH 7.5]) containing 0.1% SDS. The sedimentation profile of the monkey 28S and 18S [^{3}H]rRNA species was determined by acid precipitation of sucrose gradient fractions and scintillation counting.

Micrococcal nuclease digestions. Nuclei were usually resuspended in digestion buffers at 50×10^6 nuclei per ml. Most experiments (see Fig. 2A to C) were performed with digestion buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.1% mercaptoethanol, 1 mM $CaCl_2$, 15 mM Tris-HCl [pH 7.4]) of Hewish and Burgoyne (10), with micrococcal nuclease (6 U/10^6 nuclei) at 37°C as indicated. Digestion was stopped by addition to buffer containing 5 mM EDTA and 1% SDS. For the data shown in Fig. 2D and E, the nuclei were prepared by method 2 and digested in 0.3 M sucrose–15 mM NaCl–0.1% mercaptoethanol–0.15 mM spermine–0.5 mM spermidine–1 mM $CaCl_2$–15 mM Tris-HCl (pH 7.4), with micrococcal nuclease at 50 U/ml. The DNA was extracted from the digested samples and fractionated by electrophoresis in 2.0% (see Fig. 2A to C) or 1.5% (Fig. 2D and E) agarose gels.

Isolation and analysis of CCC DNA from liver cells. A 1.0-g portion of liver was dispersed in 10 ml of cold buffer (15 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl [pH 7.5]) in a Dounce homogenizer and extracted by the Hirt procedure (11). The Hirt supernatant fraction was extracted twice with phenol and once with chloroform and mixed with 2 volumes of ethanol to precipitate the nucleic acids. The precipitate was collected by centrifugation, dried, and dissolved in 0.5 ml of TE (10 mM Tris-HCl, 1 mM EDTA [pH 7.6]). For the analysis shown in Fig. 3B, the crude CCC DNA sample was diluted to 3.0 ml, mixed with ethidium bromide and 3.0 g of CsCl, and centrifuged for 48 h at 40,000 rpm in a Beckman SW50.1 rotor at 15°C. The gradient was fractionated by bottom puncture, and the DHBV CCC DNA species was identified by electrophoresis in an agarose gel with Southern blot assay and hybridization to a DHBV riboprobe. Ethidium bromide was removed by isopropanol extraction. Samples were subjected to thermal denaturation by immersion in boiling water for 1 min and then removed to ice to cool for 10 min. *Escherichia coli* topoisomerase I was the generous gift of Aziz Sancar; the enzyme preparation had been obtained originally from James Wang.

Two-dimensional gel electrophoresis was performed essentially as described by Peck and Wang (21). The DNA was electrophoresed in a 1% agarose gel in TBE (45 mM Tris-borate, 1 mM EDTA) at 60 V for 16 h. The gel was then soaked for 7 h in TBE containing 0.4 μM chloroquine in the dark, turned through 90°, and electrophoresed in the dark for 16 h at 60 V in TBE plus 0.4 μM chloroquine without recirculating the electrophoresis buffer.

Southern blot procedure for CCC DNA. To recover and detect the DHBV CCC DNA species in the Southern blot analyses, it was essential to soak the agarose gels in 50 mM sodium acetate (pH 4.2) for 0.5 h at room temperature and then in fresh solution for 0.5 h at 50°C. The soaks at pH 4.2 cause depurination in the DNA and thereby introduce nicks into all CCC DNA molecules. Without this preliminary depurination, the CCC DNAs renature to their duplex form after the alkaline denaturation and neutralization in the gel and are not detected after the Southern transfer to nylon membrane. The efficiency of transfer of DNA to and detection in nylon membranes by the Southern procedure was found to be independent of DNA fragment size down to at least 80 bp (the smallest size evaluated); others have reported efficient DNA transfer and retention by nylon membranes down to a size of 50 bp (2). The procedures for hybridization and washing of the blots and their analysis with DHBV probes have been described previously (19).

RESULTS

DHBV CCC DNA exists in the nucleus as a stable nucleoprotein complex. Nuclei from DHBV-infected liver were examined to determine if the viral CCC DNA was stably associated with protein. For this analysis, we used a procedure designed for the selective isolation of polyomavirus nucleoprotein complexes from bulk host chromatin (7). The nuclei from

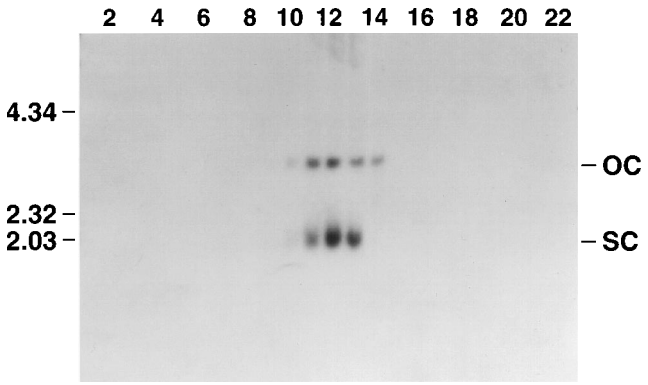

FIG. 1. Sedimentation analysis of DHBV DNA released from the isolated liver nuclei of a DHBV-infected duck. The sucrose gradient was collected by bottom puncture to yield 26 total fractions; the fractions are numbered from the bottom of the gradient to the top. The fractions were deproteinized, electrophoresed in a 1.5% agarose gel, and analyzed by Southern blot with a DHBV minus-strand [^{32}P]RNA probe. No DHBV DNA was present in fractions 23 to 26, which have been cropped from this display. The positions of standard DNA fragments and the OC and SC species of DHBV DNA are indicated.

the liver of a DHBV-infected duck were gently lysed, the cellular chromatin was preferentially removed by centrifugation, and the supernatant fraction was analyzed by zone sedimentation on a 5 to 20% sucrose gradient. Nucleic acids in the gradient fractions were extracted and subjected to agarose gel electrophoresis, and DHBV DNA was detected by the Southern blot assay with a DHBV probe. Only the two DNA species characteristic of DHBV CCC DNA preparations were detected in fractions 11, 12, and 13, of the sucrose gradient (Fig. 1). The supercoiled (SC) form of the CCC DNA migrated more rapidly in the agarose gel, and a relaxed OC DNA moved more slowly. However, both the SC and OC species cosedimented relatively homogeneously, with an apparent sedimentation coefficient of 48 $\pm$ 3S. The sedimentation profile was calibrated with 20S SV40 form I DNA and 28S and 18S rRNAs as standards. When the same supernatant fraction of the liver chromatin preparation was deproteinized prior to the sedimentation analysis, the SC and OC DNA forms were recovered in the same relative proportion and yields as shown in Fig. 1, but the sedimentation profile was quite different. The SC and OC forms no longer cosedimented at 48S (data not shown). Their sedimentation values, as deproteinized DNA molecules, were 15.5S and 12S, respectively (31). A small amount of DHBV DNA was recovered in gradient fraction 14 (Fig. 1), corresponding to a less rapidly sedimenting nucleoprotein complex, and consisted only of the OC species. This minor 41S DHBV OC DNA-protein complex probably had a less compact conformation than did the major 48S complex associated with both SC and OC DNAs. The association of the OC DNA with the less compact nucleoprotein complex strongly suggests that viral CCC DNAs are more likely to become nicked in the more open or extended complexes. For the sedimentation conditions used in the experiment described in Fig. 1, both intact DHBV virions and 100S cytoplasmic nucleocapsids were recovered from parallel sucrose gradients in the bottom fraction.

The 48S DHBV CCC DNA-protein complex probably has a similar character to the 55S nucleoprotein complex described for polyomavirus and isolated by the same protocol. The ratio of the sedimentation rates for the nucleoprotein complex and the deproteinized viral SC DNA are comparable (2.75 for polyomavirus and 3.10 for DHBV), suggesting similar but slightly different stoichiometries for the protein and DNA moieties in the two complexes. Since each 55S polyomavirus nucleoprotein complex was found to contain only a single molecule of viral SC DNA, we inferred that the 48S hepadnavirus complex similarly contained only one molecule of DHBV DNA. Another property of the polyomavirus nucleoprotein complex shared by the 48S DHBV complex was the complete susceptibility of the viral DNA to nuclease digestion. When aliquots of the sucrose gradient fractions shown in Fig. 1 were incubated with either micrococcal nuclease or bovine pancreatic DNase I and then deproteinized and reanalyzed for DHBV DNA as in Fig. 1, no viral DNA was detected (data not shown). This result was in sharp contrast to that obtained with the DHBV DNA forms present in virions or nucleocapsids, which were completely resistant to such nuclease treatments.

Micrococcal nuclease digestion analysis. The similar sedimentation profiles for the intranuclear CCC DNAs of both polyomavirus and DHBV, as stable nucleoprotein complexes, suggested that the hepadnavirus complex might have the nucleosome organization described for SV40 and polyomaviruses. Figure 2 shows the electrophoretic fractionation of DNA fragments obtained upon digestion of duck liver nuclei with micrococcal nuclease. The nuclear DNA from an uninfected duck (Fig. 2A) was efficiently cleaved by the nuclease to produce a nucleosome "ladder" of duck DNA fragments. In this digest, the mononucleosome DNA migrated as a 150- to 160-bp DNA fragment, smaller than the 200-bp DNA that was anticipated. The dinucleosome DNA, however, was approximately 400 bp, and the remainder of the ladder had a distinctive 200-bp repeat. The same pattern of nucleosome DNA was found when the analysis was performed for DNA from the liver of a DHBV-infected duck (Fig. 2B). Thus, infection by the hepadnavirus had not altered the gross organization of the duck chromatin. The DNA profiles in Fig. 2A and B were revealed by Southern blot analysis; the probe was prepared by nick translation of liver DNA from an uninfected duck. The same Southern blot shown in Fig. 2B had previously been analyzed with a DHBV probe to reveal the nuclease digestion products of the DHBV nuclear DNA (Fig. 2C). This preparation of DHBV nuclear DNA contained a high proportion of OC DNA (Fig. 2C, lane 1). Micrococcal nuclease digestion rapidly converted both the SC and OC DNA species into a prominent smear of viral DNA species from 2.0 to 0.3 kb. However, within the smear of heterogeneous viral DNA fragments, a short ladder of DHBV DNA fragments with a 150-bp repeat was discerned (lanes 3 to 9). An apparent DHBV mononucleosome DNA of approximately 150 bp was detected, as well as viral dinucleosome, trinucleosome, and tetranucleosome DNA species of 300, 450, and 600 bp, respectively. The heterogeneous and discrete DHBV DNA digestion products appeared to be derived from the SC and OC species via a duplex linear (DL) form of DHBV DNA. The heterogeneous profile of DHBV DNA shown in Fig. 2C was very reproducible. Several modifications to both the procedure for preparing the nuclei and the conditions of the nuclease digestion have been used without significant effect. Following one modified procedure, a more complete and less smeared digestion profile (Fig. 2D and E) was obtained. In this nuclear preparation, the bulk chromatin was stained with ethidium bromide and found to be already present as mononucleosomes (Fig. 2D, lane 1), evidently produced by an endogenous nuclease during the isolation of the liver nuclei (10). Subsequent incubation with micrococcal nuclease resulted in further digestion to a relatively homogeneous 150-bp DNA species. When this gel was analyzed by Southern blot with a DHBV probe, the more extensive nuclease digestion produced a much less smeared profile and revealed a series of clear bands with the distinct

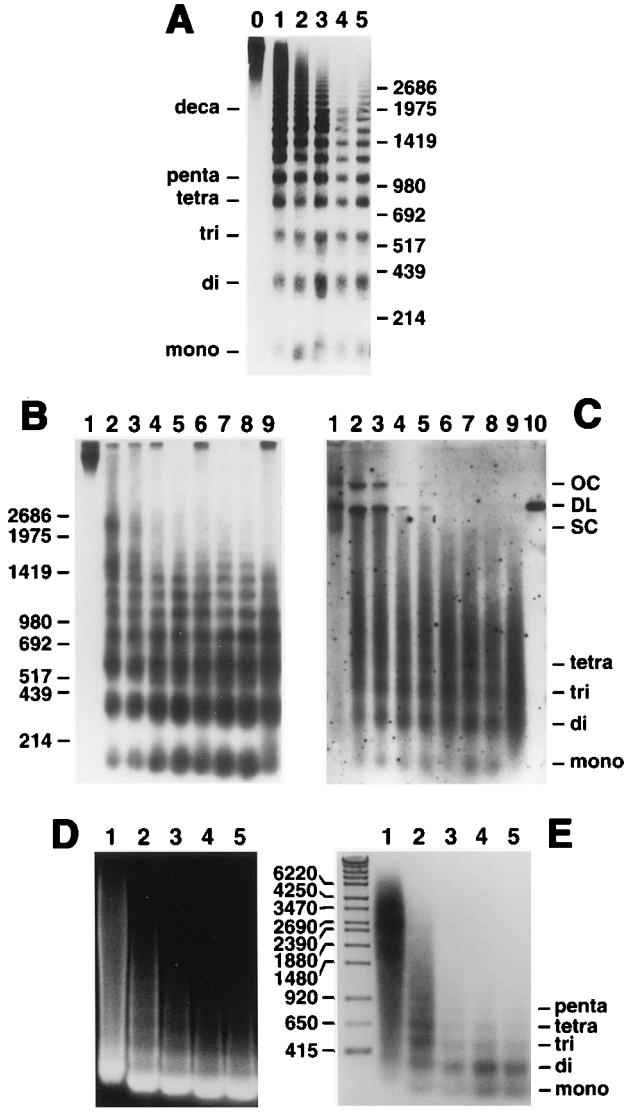

FIG. 2. Analysis of chromatin from duck liver nuclei by micrococcal nuclease digestion. (A) DNA fragments were recovered from the nuclei of an uninfected duck after 0 (lane 1), 10 (lane 2), 20 (lane 3), 40 (lane 4), 80 (lane 5), and 120 (lane 6) min of digestion respectively. The Southern blot was hybridized with a duck [^{32}P]DNA probe prepared by nick translation of liver DNA from an uninfected duck. The positions of duplex DNA fragments of known size (in base pairs) are noted; the positions of the duck liver nucleosome ladder (mononucleosome, dinucleosome, trinucleosome, etc.) are also indicated. (B) DNA fragments were from the nuclei of a DHBV-infected duck after 0 (lane 1), 5 (lane 2), 10 (lane 3), 15 (lane 4), 20 (lane 5), 30 (lane 6), 40 (lane 7), 50 (lane 8), and 60 (lane 9) min of nuclease digestion. The nuclei were prepared by method 1 (see Materials and Methods), and the duck DNA probe and DNA standards used were as in panel A. (C) Same blot as analyzed in panel B but previously hybridized with a DHBV minus-strand [^{32}P]RNA probe. Lane 10 contained a sample of cloned DHBV DNA (pBR322-DHBV p2.3) digested with *Eco*RI. Positions of the OC, DL, and SC DHBV DNA species are marked; also indicated are the positions of the DNA repeats in the viral nucleosome ladder (mononucleosome, dinucleosome, etc.). (D) This panel shows the bulk liver DNA from a DHBV-infected duck after 0 (lane 1), 5 (lane 2), 15 (lane 3), 30 (lane 4), and 45 (lane 5) min of digestion with micrococcal nuclease; the gel was stained with ethidium bromide. These nuclei were prepared by method 2 (see Materials and Methods). (E) Southern blot of the gel shown in panel D and hybridized with a DHBV [^{32}P]DNA probe. ^{35}S-labeled DNA standards are present (lane 0); the positions of the DNA repeats in the DHBV nucleosome ladder (mononucleosome, dinucleosome, etc.), are also indicated.

150-bp repeat interval (Fig. 2E, lanes 3 to 5) seen before. Specific bands were detected at 150, 300, 450, 600, 750, and 900 bp. As for the bulk duck chromatin, the DHBV DNA was already considerably digested by an endogenous nuclease prior to treatment with the micrococcal nuclease (Fig. 2E, lane 1). At the limit digest in which the bulk duck liver chromatin was reduced to 150 to 160 bp of DNA, the DHBV DNA was cleaved much less efficiently by the nucleases. The final DHBV digestion products represented approximately equal amounts of viral mononucleosome and dinucleosome DNA. The nuclease digestion studies showed that the DHBV CCC DNA was organized in part as multiple repeats of an approximately 150-bp monomer DNA that was detected in arrays as large as hexamers. Moreover, this viral DNA organization was found to be less sensitive to nuclease digestion than the bulk duck liver chromatin present in the same nuclei, which was arranged differently as nucleosomes with a 200-bp DNA repeat.

DHBV CCC DNA is a mixture of negatively superhelical topoisomers. The nucleosome is composed of 146 bp of DNA wrapped around a core histone octamer formed from two molecules each of histones H2A, H2B, H3, and H4. In bulk cell chromatin and the SV40 minichromosome, these nucleosomes are separated by approximately 50 bp of (nuclease-sensitive) linker DNA that is associated with histones H1 and H5. Clearly, the DHBV CCC DNA-protein complex must be organized somewhat differently. The micrococcal nuclease analysis for the DHBV nucleoprotein complex suggested that it is organized in part as tracts of viral hexanucleosomes which contain only short lengths of internucleosome linker DNA. Elegant studies with the SV40 system have shown a precise identity between the number of nucleosomes that are removed by protein extraction from the viral minichromosome and the number of negative superhelical turns in the recovered CCC DNA (6). The DHBV SC DNA preparation shown in Fig. 1 appeared as a homogeneous species, with no evidence for less supertwisted topoisomers migrating in the gel between the SC and OC DNA molecules. However, when a larger amount of DHBV CCC DNA was similarly analyzed, such topoisomers of the SC and OC species were detected (Fig. 3A, lane 3). All these topoisomers were cleaved by *Eco*RI digestion to form one 3,021-bp DL DNA (lane 1). The CCC nature of these topoisomers was demonstrated by heat denaturation of the DNA preparation prior to electrophoresis (33). As shown in lane 4, the several CCC DNA species all renatured and migrated with the same mobilities as in the native sample in lane 3. However, the OC DNA was stably denatured by the heat treatment to produce the two faster-migrating species designated a and b in lane 4. Species a comigrated in the gel with DNA produced by thermal denaturation of the DL DNA made by *Eco*RI digestion (compare lanes 2 and 4); species b migrated slightly faster. In a similar study in which the DHBV CCC DNA was incubated with the exonuclease III of *E. coli*, the SC DNA was unaffected while the OC DNA was digested and converted to species b (data not shown). This result demonstrated that species a and b represented the linear and circular denaturation products, respectively, expected from an OC DNA that contained a single break in one DNA strand.

To confirm the character of these isomeric CCC DNA species, they were treated with *E. coli* topoisomerase I and subjected to electrophoresis on a long agarose gel to achieve maximum resolution of the CCC topoisomers having the fewest superhelical turns. In Fig. 3B, lane 7, several topoisomers are discerned, including the OC molecule and at least eight CCC species corresponding to SC DNAs that contain two, three, four, five, six, seven, eight, and nine superhelical turns, respectively. DHBV SC DNA molecules with higher superheli-

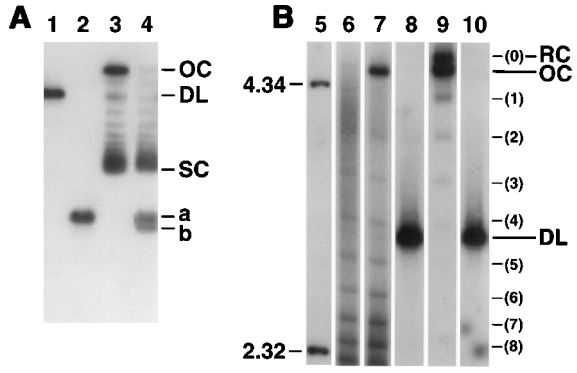

FIG. 3. Negatively supertwisted topoisomers of the DHBV SC and OC DNA species. (A) Viral CCC DNA was selectively extracted from DHBV-infected duck liver and fractionated by agarose gel electrophoresis. Viral DNA was detected in the Southern blot by a DHBV plus-strand [^{32}P]RNA probe. The DNA sample was digested with *Eco*RI (lane 1), digested with *Eco*RI and heat denatured (lane 2), untreated (lane 3), and heat denatured (lane 4). The positions of specific viral DNA species are indicated: OC, DL, SC, linear single strand (a), and circular single strand (b). (B) CCC DNA was prepared as for panel A but additionally enriched by banding in buoyant CsCl with ethidium bromide. This DNA sample was heat denatured (lane 6), untreated (lane 7), digested with *Eco*RI (lane 8), incubated with *E. coli* topoisomerase I (lane 9), or treated with topoisomerase I and subsequently digested with *Eco*RI (lane 10). ^{32}P-labeled size markers are in lane 5. The positions of the RC, OC, and DL DHBV DNA species are marked. Also indicated are the DHBV CCC DNA species containing zero, one, two, three, four, five, six, seven, and eight superhelical turns respectively. Lanes 5 through 10 were each separated in the original gel by lanes containing no DNA samples; the blank lanes have been removed for this display.

cal densities have migrated off the gel as a broad band of yet unresolved topoisomers. In lane 6, the effect of heat denaturation on this DNA preparation is shown. Only the OC DNA species failed to renature and maintain its native mobility. The denatured a and b species seen in Fig. 3A, lane 4, have also electrophoresed off the gel in Fig. 3B, lane 6, ahead of the fastest-migrating SC molecules. The *E. coli* topoisomerase I was able to almost fully relax the several DHBV SC species. A relaxed-circular (RC) DNA corresponding to a CCC DNA with zero superhelical turns was detected in the gel, migrating just behind the OC DNA (Fig. 3B, lane 9). Much of the SC DNA had been fully relaxed to the RC form; some was relaxed by nicking to the OC form. Lesser amounts of CCC DNA with one, two, three, or four supertwists were also detected in lane 9. No SC DNA that contained five or more superhelical turns was detected after the topoisomerase treatment. All of the relaxed CCC DNA species were converted by digestion with *Eco*RI to the (3,021-bp) DL DNA (lane 10). The DNA preparations used in the experiments in Fig. 3 were not made from isolated nuclei but were prepared from duck liver tissue by the Hirt extraction method (11). In this method, the viral CCC DNA species were recovered in the supernatant fraction while the replicative forms from within nucleocapsids remained in the pellet because of their covalent linkage to viral polymerase protein (37). The DNA used in Fig. 3B was further purified by banding in a buoyant CsCl density gradient containing ethidium bromide. The CCC DNA fraction was thus separated from the other nucleic acids present; removal of the non-CCC DNA greatly facilitated the topoisomerase reactions.

E. coli topoisomerase I, unlike its eukaryotic counterpart, can efficiently relax only CCC DNA molecules that contain negative superhelical turns (34). Thus, the data in Fig. 3 demonstrate conclusively that the deproteinized DHBV CCC DNA is actually a heterogeneous population of negatively supertwisted CCC DNA molecules and is consistent with their likely derivation from a similarly heterogeneous population of DHBV minichromosomes in situ. Some of these DHBV minichromosomes would contain as few as two nucleosomes, while others would have more than nine.

To resolve all of the topoisomeric species present in DHBV CCC DNA, two-dimensional agarose gel electrophoresis was performed. In this method, the second dimension of electrophoresis was carried out in the presence of chloroquine. The binding of chloroquine to CCC DNA introduced positive superhelical turns in the DNA-chloroquine complex (25, 35). We reasoned that a DHBV CCC DNA (3,021 bp) completely associated into nucleosomes would contain either 15 nucleosomes (200 bp per nucleosome) or 20 nucleosomes (150 bp per nucleosome). The chloroquine concentration was titrated to add 10 positive superhelical turns to the DHBV DNA for electrophoresis in the second dimension, so that any CCC species that originally had 10 to 20 negative superhelical turns would be resolved. The DNA used in this analysis was prepared from infected liver by the Hirt extraction method, and after electrophoresis, the agarose gel was analyzed by the Southern blot procedure and hybridized with a DHBV probe. The analysis showed clearly (Fig. 4) that the DHBV CCC DNA is a heterogeneous population of topoisomers that contains 0 to 20 negative superhelical turns when first extracted. All 21 CCC DNA topoisomers were represented in the DNA preparation, although those with small numbers (less than four) of superhelical turns were the least abundant. Over 80% of the DHBV SC DNA was resolved into two subpopulations of CCC DNA molecules. One subpopulation had 18, 19, or 20 superhelical turns per SC DNA molecule, while the other had 8, 9, 10, or 11. The former population was probably derived from DHBV minichromosomes that were essentially fully organized into nucleosomes, with a 150-bp DNA repeat and, by inference, only a small amount of linker DNA. The second population, roughly as abundant as the first, presumably came from viral minichromosomes in which approximately half of the DHBV DNA was associated into nucleosomes.

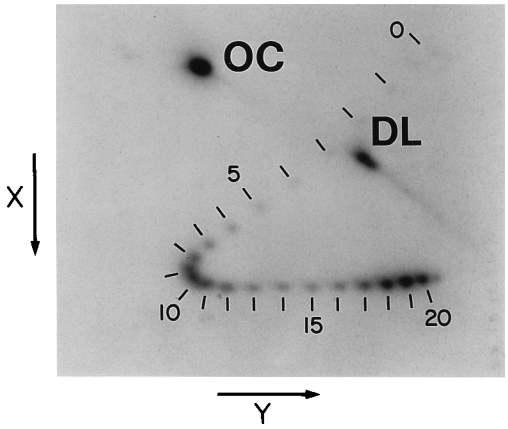

FIG. 4. Fractionation of DHBV CCC DNA topoisomers by two-dimensional agarose gel electrophoresis. The viral DNA was prepared by selective extraction from DHBV-infected duck liver (11). The gel was analyzed by Southern blot and hybridization to a DHBV minus-strand [^{32}P]RNA probe. The full autoradiogram was cropped to show only that portion of the gel containing DHBV DNA species. The X direction marks the first dimension of electrophoresis; the Y direction is the second dimension when chloroquine was present. The positions of the OC and DL DHBV DNAs are labeled. Also marked are the positions in the gel for each of the 21 CCC topoisomers, designated 0 to 20, to indicate the superhelicity of each CCC DNA species.

DISCUSSION

The organization and protein associations of the hepadnavirus CCC DNA in situ were studied by sedimentation, micrococcal nuclease digestion, and DNA superhelicity analyses. These three lines of investigation indicate that the DHBV CCC DNA is stably associated with proteins in the nuclei of infected liver cells. Moreover, they provide very strong indirect evidence that the viral nucleoprotein complex is indeed a minichromosome composed of classical nucleosomes but in arrays that are atypical of most chromatin. Direct electron microscopy and protein composition studies are technically difficult because of the low copy number of these viral complexes in infected cells and the significant background of residual host chromatin still present in preparations of the viral nucleoprotein complexes.

It was recently reported (1) that the human hepatitis B virus (HBV) CCC DNA is a viral minichromosome in the cultured hepatoblastoma cell line HepG2.2.15 (23). The inferred character of the HBV minichromosome is different from that proposed here for DHBV. The analyses of the in vitro system were complicated, however, by the presence of comparable yet unresolved amounts of both CCC and integrated HBV DNA sequences in the chromatin from these nuclei.

The superhelicity of the DHBV CCC DNA species ranges from 0 to 20 supertwists per molecule, with all 21 topoisomers present in each DNA preparation. Both the specificity of the topoisomerase and the effect of the chloroquine binding confirm that the supertwists are negative. The usual profile of viral CCC DNA on agarose gel electrophoresis, as typified in Fig. 1, is thus seen to be misleading. The SC band is really a mixture of all the CCC topoisomers ranging from 8 to 20 supertwists, which remain unresolved by the electrophoresis. The less supertwisted topoisomers are not detected in the sucrose gradient analysis because of their low abundance. The OC DNA species can be distinguished from the RC CCC DNA that contains zero supertwists, and it probably arises by artifactual random nicking of CCC DNA molecules throughout the isolation and manipulation of the DNA. We have no evidence to indicate that the OC DNA exists as such in situ.

The DHBV genome contains 3,021, 3,024, or 3,027 bp, depending on the strain of the virus (27). Thus, a viral CCC DNA with 20 supertwists has 151 bp of DNA per supertwist; this amount of DNA could derive from a classical nucleosome containing 146 bp of DNA with an additional 5 bp of linker DNA. The calculated value of 151 bp correlates with the apparent size for the viral mononucleosome DNA (150 to 160 bp) extrapolated from the micrococcal nuclease digests. It is also concordant with the 150-bp repeat observed for the viral DNA ladders in the same analyses. In fact, a viral minichromosome composed of 20 nucleosomes each containing 146 bp of DHBV DNA is precisely compatible with the observed maximal superhelicity and the nuclease digestion profile.

The DHBV CCC DNA species containing less than 20 supertwists have correspondingly more than 151 bp DNA per supertwist. For example, topoisomers with 15 and 8 supertwists correspond to 201 and 378 bp of viral DNA per supertwist, respectively. If such repeats for these (and the other 17 SC) topoisomers represent other stable protein-DNA structures present in the DHBV nucleoprotein complex, they might contribute their periodicities to the nuclease digestion profile of the viral DNA and in aggregate perhaps to its smeared character. Since only the 150-bp DNA repeat ladder is detected for the viral DNA present in nuclei, such other repeated structures either do not exist or are totally susceptible to the nuclease digestion. The (5 to 10%) yield of viral DNA recovered in the 150-bp repeats (from Fig. 2E) does roughly correspond to the prevalence of the CCC DNA with 20 supertwists (from Fig. 4), making this latter explanation tenable. However, the data are also compatible with the more likely hypothesis that the less supertwisted topoisomers are derived from minichromosomes which have one or more tracts of classical nucleosomes with a 150-bp repeat and contain correspondingly more linker DNA (Fig. 5). If the linker DNA was extended and protein free, much of the viral chromatin would be more sensitive to nuclease digestion than the bulk chromatin and not resistant as observed. It seems likely, therefore, that the viral linker DNA is associated with proteins that limit and retard its cleavage by nucleases. An irregular distribution of such DNA-bound proteins might explain the heterogeneous smear of viral DNA found in the digests. The interpretation of the superhelicity data given here is based on the key assumption that viral CCC DNA molecules are torsionally relaxed as nucleoprotein complexes in situ prior to extraction from infected cells. Thus, a CCC topoisomer with 14 supertwists is assumed to derive only from a minichromosome composed of 14 nucleosomes and would not represent a minichromosome in situ with 9 nucleosomes plus 5 negative supertwists of its DNA.

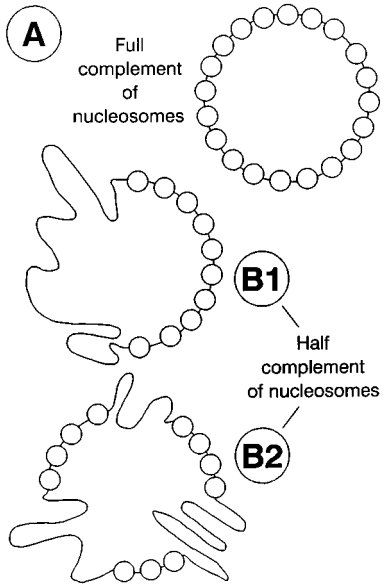

FIG. 5. Schematic representations of the DHBV minichromosome. (A) The minichromosome is depicted with 96.7% (= [146 × 20 ÷ 3,021] × 100%) of the DHBV DNA compacted as 20 nucleosomes, each spaced on average by 5 bp of linker DNA. (B) A second DHBV minichromosome is shown with approximately 50% of the viral DNA organized into 10 similarly spaced nucleosomes and the remaining 50% as extended (not shown correctly to scale) viral DNA. The 10 nucleosomes can be arrayed as a single tract (B1) or in a few smaller groups (B2).

The model for the DHBV minichromosome, depicted schematically in Fig. 5, embodies the calculated 151-bp viral DNA as the predominant nucleosomal repeat. Unlike the constant amount of DNA wound on each nucleosome, the linker DNA probably can vary, and 5 bp represents the average spacing between adjacent nucleosomes. The significant presence of viral dinucleosome DNA in the limit nuclease digest (Fig. 2E) may represent mononucleosome pairs that are joined by the least (0- to 4-bp) linker DNA. The same model also predicts that the nuclease digests should contain viral nucleosome repeats up to 20 units in length, yet only hexanucleosome arrays are reported (Fig. 2E). Viral nonanucleosomes have been detected in other gels, and larger strings of viral nucleosomes are

probably present in these digests at low concentrations but were not detected.

No specific proteins have been identified as components of the DHBV minichromosome. The sucrose gradient fractionation shown in Fig. 1 has a high background of host chromatin and contained no detectable amounts of viral core and envelope proteins, indicating little contamination by cytoplasmic components. Gradient fractions did contain both nonhistone proteins and histones, but none were found to cosediment concordantly with the 48S complex (data not shown). However, we infer that the DHBV minichromosome contains the four core histones plus a variable amount of uncharacterized DNA-binding proteins associated with much of the linker DNA. The method used to isolate the 48S DHBV nucleoprotein complex (Fig. 1) releases the analogous polyomavirus and SV40 complexes as 55S minichromosomes, depleted of their histone H1 by exposure to salt (9). The stable and relatively homogeneous 48S complex for DHBV is thus unlikely to contain H1. Of course, the main function of the nucleosome is to compact DNA within the nucleus. The 55S (H1-depleted) minichromosomes of polyomavirus and SV40 sediment at 2.75 times the rate of their SC DNA molecules. For DHBV, this ratio is 3.1 and indicates an even greater compaction for the hepadnavirus DNA in its minichromosome. This is consistent for the more superhelical DHBV topoisomers, which have a greater proportion of their CCC DNA compacted into nucleosomes than the polyomavirus and SV40 DNAs do. However, the DHBV minichromosomes composed of fewer nucleosomes and more linker DNA also sediment at 48S. The proteins bound to the extensive linker DNA in these complexes must therefore not only protect it from nuclease digestion but also confine it in a compact conformation.

It is a striking observation, and quite distinct from the SV40 paradigm, that the same hepatocyte nuclei contain both 150- and 200-bp DNA ladders in the DHBV and duck chromatins, respectively. Whether this distinct organization has profound ramifications for the viral chromatin remains unclear. Certainly, duck hepatocytes are not deficient in histone H1. This feature of the viral chromatin may simply reflect the different times at which both chromatins are formed. The hepatocyte chromatin was condensed during the S phase prior to the last mitosis. It has been proposed that this might occur in a hepatic stem cell that is resistant to hepadnavirus infection (12). Thus, DHBV infection of the descendant hepatocytes would subsequently lead to the condensation of the viral chromatin, perhaps when the 200-bp DNA ladder can no longer be formed. An alternative hypothesis would correlate the 200-bp repeat of the duck chromatin with the semiconservative replication scheme of its DNA. The DHBV minichromosome with its distinctive 150-bp repeat represents a similarly unique "replicon" that does not undergo semiconservative DNA replication. However, the cauliflower mosaic virus CCC DNA, like hepadnavirus CCC DNA, is not replicated semiconservatively, yet the caulimovirus minichromosome displays the same 200-bp repeat as the host plant chromatin (20).

Far more provocative are the possible biological and functional implications for the different subpopulations of the DHBV minichromosome. Are the fully-chromatinized and half-chromatinized minichromosomes (Fig. 5) in a dynamic equilibrium with each other, with viral CCC DNAs shuttling between the two populations? Or are they separate species committed to one or the other composition by distinct modifications to core histone molecules or by the binding of specific nonhistone proteins? Certainly, it is plausible that the two subpopulations might be distinguished in both a differential stability and their transcriptional activity. Minichromosomes containing 8 to 11 nucleosomes might represent the more active chromatin and be the templates for the most abundant viral transcripts. Possibly, all of the DHBV promoters are expressed in the same template molecule; alternatively, individual minichromosomes might be dedicated to the RNA synthesis from only a single viral promoter, perhaps determined by its chromatin organization.

The characterization of the DHBV chromatin assembled in this report constitutes compelling proof that it is indeed organized in situ as a viral minichromosome. Moreover, these molecules display both a distinctive nucleosome repeat and a heterogeneity that may provide useful insights into the organization and regulation of all chromatin. It will be interesting to extend these observations to HBV, which has a slightly larger genome and, if organized like DHBV chromatin, could accommodate one extra (151-bp) nucleosome in an HBV minichromosome. Thus, an HBV minichromosome might contain a maximum of 21 nucleosomes and give rise to some HBV CCC DNA molecules with 21 superhelical turns. Additionally, if HBV chromatin in infected patients is characterized by an atypical organization, it could represent a unique and potentially useful target for novel antiviral therapies.

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