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Closed Circular Viral DNA and Asymmetrical Heterogeneous Forms in Livers from Animals Infected with Ground Squirrel Hepatitis Virus

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To identify possible intermediates in the replication of ground squirrel hepatitis virus, we characterized the major forms of intracellular virus-specific DNA in the livers of infected ground squirrels. A variety of DNA species were found: covalently closed circular molecules, relaxed circular molecules, and a heterogeneous collection of molecules that migrated ahead of closed circular DNA during agarose gel electrophoresis. The heterogeneous DNA was at least partly single stranded, consisted of minus strands in a greater than eight-fold mass excess of plus strands, and was tightly associated with protein.

Ground squirrel hepatitis virus (GSHV) is a member of the newly described group of hepatitis B-like viruses found first in humans and subsequently in woodchucks, ground squirrels, and ducks (8, 10, 22). The unusual genome of these viruses, as first defined with DNA extracted from virions of human hepatitis B virus (HBV), is a relaxed circular molecule (15), with its circularity maintained by hydrogen bonding of 5′ cohesive termini of 250 to 300 base pairs in length (17). The molecule is partially single stranded, with the minus strand having a uniform length of 3,200 base pairs and the plus (sense) strand varying in length in different molecules (5, 7, 21, 24). A DNA polymerase in the nucleocapsid core of the virion can elongate the incomplete plus strand to make a double-stranded relaxed circular molecule of 3,200 bp (5–7, 21). In addition, protein is covalently bound to the 5′ end of the minus strand (3).

Study of the replication and expression of this unusual type of genome has been limited, since the hepatitis B viruses have not been propagated in tissue culture. Efforts to decipher the virus life cycle have therefore focused upon viral components extracted from the livers of infected clinical and experimental subjects. Our laboratory is using GSHV as a model for replication of hepatitis B viruses. We have transmitted GSHV by inoculating susceptible Beechey ground squirrels with serum from naturally infected animals (2). After a 6- to 12-week latent period, about 60% of the inoculated animals manifest infection by the appearance in the blood of GSHV surface antigen, the outer coat protein of the virus. About 15% of the animals have continued to produce GSHV surface antigen for more than 1 year. GSHV demonstrates marked hepatotropism: viral DNA can be detected in significant quantities only in the liver. We and others have shown that GSHV virion DNA resembles HBV DNA in size and structure (1, 8); furthermore, GSHV DNA also has protein linked to the minus strand (1). Restriction site polymorphisms exist among natural isolates of GSHV, and we have cloned the genomes of two variants, strains 27 and 36, in *Escherichia coli* (1).

In this paper we identify the major GSHV-specific forms of DNA in the livers of experimentally infected ground squirrels: covalently closed circular molecules, open circular molecules, and a heterogeneous collection of DNA associated with protein and containing minus strands in an 8- to 10-fold mass excess of plus strands. These results are in agreement with those of a similar analysis of intracellular forms of duck hepatitis B virus (DHBV) (9, 12) and are consistent with the recently proposed model of Summers and Mason for the replication of hepatitis B viruses (20; see below).

MATERIALS AND METHODS

Squirrels. Beechey ground squirrels were trapped in the wild in Palo Alto, Calif., and infected with GSHV as described previously (2). In all experiments but one, liver tissue was obtained from squirrels that had been inoculated with virus 8 to 12 weeks previously; the serum of all animals studied here showed high levels of GSHV surface antigen in the heterologous radioimmunoassay for HBV surface antigen (Ausria II; Abbott Laboratories, North Chicago, Ill.). In one experiment noted in the text (see Fig. 8), liver tissue was obtained from animals that had been infected for

more than 1 year as judged by persistently positive assays for GSHV surface antigen. Liver tissue was obtained by open biopsy or autopsy, and specimens were immediately frozen in a dry ice-acetone bath and stored at −70°C until used.

Preparation of virion DNA. Virions were concentrated from serum, and virion DNA was repaired by the endogenous and avian myeloblastosis virus polymerase reactions and extracted as described previously (1, 2).

Preparation of high-molecular-weight DNA from ground squirrel livers. Whole cell DNA was extracted by a modification of a procedure described previously (14). Frozen tissue was minced and homogenized. Proteinase K (200 μg/ml; Merck Sharp & Dohme, West Point, Pa.) and sodium dodecyl sulfate (SDS) (1%) were added, and the solution was incubated at 50°C for 3 h and extracted with phenol-chloroform (1:1) until the aqueous phase cleared. The solution was extracted two times with chloroform, and the nucleic acid was precipitated with ethanol and suspended in TE (10 mM Tris-hydrochloride (pH 8.1)–1 mM Na_3 EDTA). SDS (1%) was added, and the suspended nucleic acid was digested with RNase (100 μg/ml) at 37°C for 1 h, redigested with proteinase K, and reextracted with phenol-chloroform. The DNA was precipitated with ethanol and resuspended in TE.

In one experiment noted in the text, DNA samples were phenol extracted without prior proteinase K digestion. In that experiment, tissue was minced and homogenized in buffer, SDS (2%) and β-mercaptoethanol (2%) were added, and the solution was incubated at 60°C for 30 min. The mixture was deproteinized with phenol-chloroform, and the nucleic acids were precipitated with ethanol, dissolved in TE, digested with RNase as described above, reextracted with phenol-chloroform, ethanol precipitated, and resuspended in TE.

Restriction endonuclease and single strand nuclease reactions. Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.) and Bethesda Research Laboratories (Bethesda, Md.). DNA samples of 10 μg each were digested in a volume of 200 to 250 μl for 12 to 16 h under the conditions recommended by the manufacturers with an excess of enzyme. Digestions were monitored for completion by adding 500 ng of supercoiled plasmid DNA to a small portion of the reaction mixture, incubating this portion in parallel, and detecting the conversion of plasmid DNA to a linear form by agarose gel electrophoresis and ethidium bromide staining. Mung bean nuclease was purchased from P-L Biochemicals (Milwaukee, Wis.), and reactions were performed for 30 min at 37°C in the following buffer: 50 mM sodium acetate, 250 mM sodium chloride, 4.5 mM zinc sulfate, 2% glycerol. Reactions were stopped by the addition of 2 μl of solution containing 20% SDS and 0.5 M EDTA. S1 nuclease was purchased from Bethesda Research Laboratories, and digestions were performed for 20 min at 37°C with the same buffer as described for mung bean nuclease plus calf thymus DNA at a concentration of 100 μg/ml. Reactions were stopped as described above.

Cesium chloride-propidium diiodide equilibrium gradients. Sample liver DNA (200 μg) and simian virus 40 (SV40) marker DNA (50 ng) were dissolved in 6.1 ml of TE, and 5.63 g of solid cesium chloride and 0.4 ml of propidium diiodide at 6 mg/ml were added to each sample. Gradient samples of 9 ml each were spun at 33,000 rpm for 70 h at 20°C in a Beckman type 40 rotor. Fractions of 0.5 ml each were collected from the bottom of each gradient, diluted with water, precipitated with 2 volumes of ethanol, and resuspended in TE before analysis by agarose gel electrophoresis. A portion of DNA recovered from certain gradient fractions was subjected to enzymatic digestion; this DNA was extracted with isoamyl alcohol to remove propidium diiodide before enzymatic digestion.

Analysis of cellular DNA. Liver DNA was analyzed by electrophoresis through agarose gels followed by transfer to nitrocellulose filters (19), hybridization with radioactive probes, and autoradiography. These procedures have been described in detail previously (13).

Dot blot hybridizations. Dot blot hybridizations were performed as described previously except that dextran sulfate was not used in the filter hybridization step (2, 23).

Preparation of molecular hybridization probes. A GSHV probe representative of the entire genome, $GSHV_{rep}$, was made with GSHV DNA (strain 27) cloned in pBR328 at the unique *Eco*RI site in the viral genome (1). ^{32}P-labeled probes were synthesized by copying this template with avian myeloblastosis virus polymerase and oligomeric calf thymus primers (18); the specific activity was 1×10^8 to 2×10^8 cpm/μg of DNA.

To isolate the two strands of GSHV DNA for preparation of strand-specific probes, we subcloned the entire GSHV insert from the pBR328 clone (1) in both orientations into the *Eco*RI site of the single-stranded bacteriophage M13mp8. M13mp8 was obtained from Bethesda Research Laboratories, and cloning and preparation of M13 bacteriophage DNA were performed as recommended by the manufacturer (11). Strand-specific probes were made as described by Mason et al. (9). [^{32}P]DNA (specific activity, 1×10^8 to 3×10^8 cpm/μg of DNA) complementary to wild-type M13mp8 phage DNA was synthesized with avian myeloblastosis virus polymerase with calf thymus DNA as the primer. The reaction products were denatured with heat (100°C for 5 min) and hybridized to M13mp8 recombinant phage DNA carrying a GSHV insert; this procedure generates labeled, double-stranded M13 sequences, but the GSHV insert remains single stranded. Labeled DNA (10 to 20 ng; 2×10^6 cpm) was hybridized to 20 to 40 ng of unlabeled recombinant phage containing the GSHV insert for 2 h at 68°C in 5 × SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The hybridized DNA was then diluted into 3 to 5 ml of hybridization solution (2).

The strand specificities of two recombinant clones, PRR 18 and PRR 12, were determined by using these clones as probes for native unrepaired virion DNA. Unrepaired DNA was applied to nitrocellulose filters by the dot blot technique (23) and allowed to hybridize to each clone. Because the minus strand of native virion DNA is partially single stranded, it will hybridize to a recombinant phage containing a plus-strand insert; phage containing a minus-strand insert is not expected to hybridize to native virion DNA. As seen in Fig. 1, clone PRR 18 hybridized to native virion DNA and therefore contains a plus-strand insert; clone PRR 12 did not hybridize to native virion DNA and therefore contains a minus-strand insert. In parallel

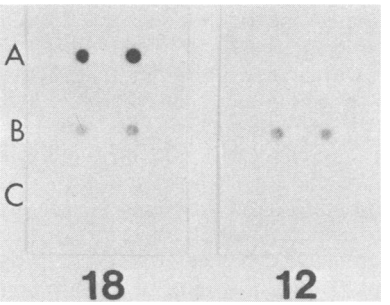

FIG. 1. Strand specificity of M13mp8 clones. Unrepaired native virion DNA was applied in duplicate to two nitrocellulose filters. Ten picograms each of native and denatured cloned GSHV DNA were applied as control samples. The filters were hybridized to ^{32}P-probes prepared with clones PRR 18 (left) and PRR 12 (right) as described in the text. (A) Native virion DNA; (B) denatured cloned DNA; (C) native cloned DNA.

controls, denatured cloned GSHV DNA hybridized to probes prepared from both PRR 18 and PRR 12, whereas neither probe detected native cloned GSHV DNA.

RESULTS

Intracellular GSHV DNA. We examined the structure of GSHV-specific DNA in 10 livers from infected animals by agarose gel electrophoresis, DNA transfer, and hybridization to cloned ^{32}P-GSHV DNA. The characteristic pattern, observed with all samples, is illustrated in Fig. 2. We found two discrete species, one comigrating with repaired open circular virion DNA (lane M), and another migrating more rapidly in a position appropriate for superhelical closed circular DNA of approximately 3.2 kilobases (kb). In addition, there was a large amount of heterogeneous DNA, most of which migrated faster than the two discrete forms.

Covalently closed circular viral DNA in infected livers. The mobilities of the two discrete bands of GSHV-specific DNA suggested that they might represent unit-length open and closed circular duplex viral DNAs. To test for superhelical closed circular (form I) DNA, total cellular DNA from the liver of an animal infected with GSHV strain 36 was fractionated by equilibrium centrifugation in a cesium chloride-propidium diiodide (CsCl-PI$_2$) gradient; unlabeled SV40 form I DNA was added to the same gradient as an internal marker. DNA from the gradient fractions was then subjected to agarose gel electrophoresis; after ethidium bromide staining to detect marker SV40 DNA, the DNA was transferred to nitrocellulose paper and annealed to ^{32}P-GSHV DNA (Fig. 3). The rapidly migrating discrete GSHV species cosedimented with SV40 form I DNA, suggesting that it is indeed superhelical. Also seen in the gradient (Fig. 3) was the more slowly migrating discrete GSHV species (most prominent in fraction 11), which cosedimented with SV40 form II DNA, and the heterogeneous forms, which were dispersed throughout most of the gradient and were partially fractionated by density. This fractionation of heterogeneous forms will be discussed below.

To test further whether the rapidly migrating discrete species was covalently closed circular DNA, we subjected GSHV-infected liver DNA and CsCl-PI$_2$-purified DNA to enzymatic digestion. Total cellular DNA from the liver of an animal infected with GSHV strain 27 was digested with *Bgl*II and *Hin*dIII (Fig. 4A, lanes 1 and 2), which do not cleave viral DNA, and with *Eco*RI and *Pvu*II (Fig. 4A, lanes 3 and 4), both of which cleave the genome of GSHV strain 27 at one site (1). If the two bands seen before digestion represented covalently closed circular and nicked open circular DNA, enzymes that cleave viral DNA once would convert these two species to one that would migrate slightly ahead of open circular DNA, in a position appropriate for linear (form II) DNA. These are the results seen in Fig. 4A. We also analyzed DNA from the same liver isolated from CsCl-PI$_2$ gradient fractions containing putative covalently closed GSHV molecules, with the same result (Fig. 4B). In addition, DNA from the liver of an animal infected with GSHV strain 36 was isolated from the CsCl-PI$_2$ gradient shown in Fig. 3 and digested with *Eco*RI, which cleaves the genome of this strain at two sites, producing

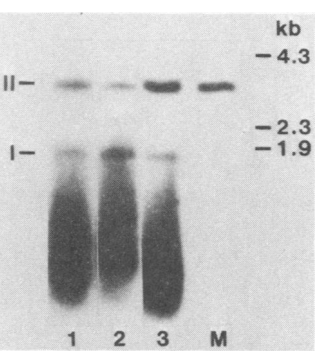

FIG. 2. GSHV DNA in the livers of infected squirrels. Twenty micrograms of DNA from the livers of GSHV-infected squirrels was digested with *Bam*HI (an enzyme which does not cleave viral DNA), electrophoresed through a 1% agarose gel, transferred to nitrocellulose, and annealed with a ^{32}P-labeled GSHV$_{rep}$ probe as described in the text. Lanes 1 through 3 show DNA from the livers of three infected squirrels. Lane M shows fully repaired open circular GSHV virion DNA (3.2 kb) as a marker. Linear size markers are shown at the right in kb, and the positions of form I and form II DNA of 3.2 kb are indicated on the left.

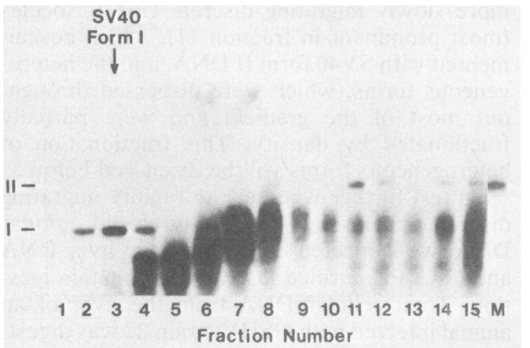

FIG. 3. Analysis of GSHV DNA from infected livers by CsCl-PI_2 equilibrium centrifugation. A total of 200 μg of total liver DNA from an animal infected with GSHV strain 36 and 50 ng of SV40 DNA were centrifuged in a CsCl-PI_2 equilibrium gradient as described in the text. One-half of the DNA recovered from each fraction was electrophoresed on a two-tier 0.8% agarose gel, transferred to nitrocellulose, and annealed to a ^{32}P-labeled $GSHV_{rep}$ probe as described in the text. Lane M denotes repaired GSHV DNA used as a marker. SV40 form I DNA, detected visually by ethidium bromide staining of the gel, was found in fraction 3 of the gradient. The positions of GSHV form I and form II DNA are denoted on the left.

fragments of 1,500 and 1,700 base pairs. Two fragments of viral DNA of those sizes were produced upon digestion of DNA from fractions containing closed circular species (Fig. 4C). We also treated the pooled putative form I DNA isolated from the CsCl-PI_2 gradient shown in Fig. 3 with increasing quantities of the single strand-specific mung bean nuclease. Treatment with this enzyme, which can nick covalently closed circular DNA to form open circular DNA, resulted in conversion of increasingly large proportions of the rapidly migrating molecules to a species comigrating with open circular repaired virion DNA (Fig. 4D), confirming that the rapidly migrating molecules were covalently closed circular DNA. As a control, SV40 form I DNA was digested in parallel with the same number of units of mung bean nuclease, converting approximately the same proportions of SV40 DNA from form I to form II.

Partial single-stranded nature of and excess of minus strands in heterogeneous DNA in infected livers. Most of the heterogeneous DNA seen in Fig. 2 migrated faster upon agarose gel electrophoresis than did covalently closed circular DNA. After CsCl-PI_2 centrifugation (Fig. 3) these forms were seen throughout the gradient but were partially fractioned by density, so that the molecules migrating most rapidly upon agarose gel electrophoresis sedimented to positions of greatest density in the gradient. This fractionation by density suggested that the most rapidly migrating forms were at least partially single stranded (4). We therefore investigated whether the heterogeneous DNA consisted of partially

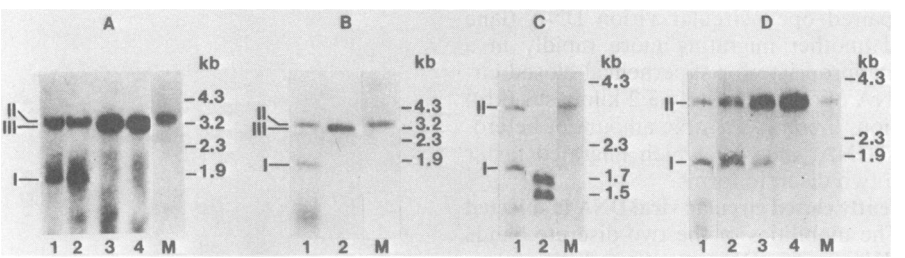

FIG. 4. Enzymatic tests to demonstrate the presence of covalently closed circular molecules. Samples of unfractionated, GSHV-infected liver DNA (A) or CsCl-PI_2-purified DNA (B through D) were subjected to enzymatic digestion and then electrophoresed on 1% agarose gels, transferred to nitrocellulose, and annealed to ^{32}P-labeled $GSHV_{rep}$ as described in the text. In each panel, lane M shows a marker of open circular fully-repaired GSHV virion DNA, the sizes of linear DNA species (in kb) are marked on the right, and the positions of forms of GSHV DNA are indicated on the left. (A) Ten micrograms of total liver DNA from a GSHV strain 27-infected squirrel were digested to completion with *Bgl*II (lane 1), *Hin*dIII (lane 2), *Eco*RI (lane 3), and *Pvu*II (lane 4). (B) Whole cellular DNA from the liver of a squirrel infected with GSHV strain 27 was analyzed on a CsCl-PI_2 equilibrium gradient as described in the text and the legend to Fig. 3. Putative covalently closed GSHV DNA was identified as a rapidly migrating band in two fractions cosedimenting with SV40 form I DNA; DNA was recovered from these fractions as described in the text and pooled. Twenty percent of the pool was electrophoresed without further treatment (lane 1), and 20% was digested to completion with *Eco*RI (lane 2) before electrophoresis. (C) Putative form I DNA from the liver of a squirrel infected with GSHV strain 36 was recovered from fractions 2 and 3 of the gradient shown in Fig. 3. Twenty percent of the pooled DNA was electrophoresed without further analysis (lane 1), and 20% was digested to completion with *Eco*RI before electrophoresis (lane 2). (D) Equal portions (20%) of the pooled DNA described in (C) were digested with increasing amounts of mung bean nuclease. (Lane 1) undigested DNA; (lanes 2, 3, and 4) DNA digested with 1, 10, and 100 U of mung bean nuclease, respectively. The same sample of untreated putative form I DNA is depicted in lane 1 of both (C) and (D).

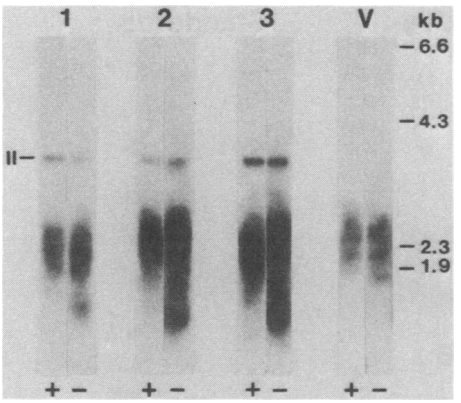

FIG. 5. Annealing of DNA from GSHV-infected livers to strand-specific probes. Ten micrograms of DNA from three livers (panels 1, 2, and 3) of GSHV-infected squirrels were digested with *Bam*HI, electrophoresed on a 1% agarose gel, transferred to nitrocellulose as described in the text, and annealed to the ^{32}P-labeled probe PRR 12 that detects plus strands (lanes marked +). The probe was removed from the filter, and the filter was reannealed to the ^{32}P-labeled probe PRR 18 that detects minus strands (lanes marked −). Panel V shows unrepaired virion DNA. Linear size markers are shown at the right in kb, and the position of GSHV form II DNA is denoted on the left.

single-stranded viral DNA or merely smaller duplex DNA and, if partly single stranded, whether one strand was more abundant than the other.

As a first test for the presence and polarity of single-stranded viral DNA, DNA from three infected livers was examined by Southern blot analysis and hybridization to each of the strand-specific viral probes (Fig. 5). This experiment showed that minus-strand DNA (detected by plus-strand probe PRR 18 [Fig. 5]) was present in greater quantities than was plus-strand DNA (detected by minus-strand probe PRR 12]Fig. 5]) and that a significant portion of the minus-strand DNA migrated more rapidly upon agarose gel electrophoresis than did any detectable plus-strand DNA. The detection of minus-strand DNA in a low-molecular-weight region of the gel where no plus-strand DNA was detected suggested that there might be fully single-stranded minus-strand DNA in GSHV-infected livers; it is also possible that the minus-strand DNA detected in this region was largely single stranded but was paired to short plus strands which were not detected by the techniques used here. The amount of heterogeneous viral DNA and its pattern of migration varied somewhat among different liver samples; however, an excess of minus-strand DNA was seen in each of the livers tested.

To confirm the presence of at least partially single-stranded viral DNA and to estimate the amount of each strand present, we used the dot blot hybridization technique (23). Serial dilutions of native and denatured GSHV-infected liver DNA were applied to nitrocellulose filters and annealed to each of the strand-specific probes (Fig. 6). Viral DNA in infected livers annealed to a probe detecting minus strands without denaturation of the test samples (Fig. 6B); however, plus-strand DNA could only be detected after denaturation (Fig. 6C and D). These results indicated that GSHV-infected livers contained minus-strand DNA that was at least partially single stranded; plus-strand DNA, by contrast, appeared to be fully duplex. An estimate of the amounts of each strand present in infected livers was made by comparing the intensity of autoradiographic signals obtained with serial dilutions of denatured GSHV-infected liver DNA to the intensity of signals obtained with known amounts of cloned GSHV DNA applied to the same filters as standards (Fig. 6A and C). Six different livers, three of which are shown in Fig. 6, showed a range of 20 to 320 pg of GSHV minus-strand DNA per microgram of liver DNA; about 2 to 40 pg of plus-strand DNA per microgram of liver DNA was detected. Thus, there was approximately 8 to 10 times as much minus-strand DNA as plus-strand DNA present in infected livers, indicating that 80 to 90% of intracellular minus-strand sequences are single stranded. This result is in accord with the finding that denaturation of the sample did not produce a notable increase in hybridization to a probe that detected the minus strand (Fig. 6A and B).

To confirm that minus-strand DNA in infected livers is at least partially single stranded but that plus-strand DNA is entirely in duplex form, we digested DNA from an infected liver with increasing quantities of S1 nuclease and then analyzed the DNA by the dot blot technique and hybridization to each of the strand-specific probes. As seen in the previous experiment, minus-strand DNA was detected both before and after denaturation (Fig. 7A, lanes 1 and 2); plus-strand DNA was detected only after denaturation (Fig. 7B, lanes 1 and 2). The minus-strand DNA displayed a large degee of S1 nuclease sensitivity (Fig. 7A, lanes 1 and 2), whereas plus-strand DNA was S1 nuclease resistant (Fig. 7B, lane 2). Control samples of native and denatured cloned viral DNA digested in parallel with S1 nuclease verified that native cloned viral DNA is resistant to S1 nuclease and that denatured cloned viral DNA is highly sensitive in this assay (Fig. 7, lanes 3 and 4). The small proportion of minus-strand DNA that was resistant to S1 nuclease appeared to be equal to

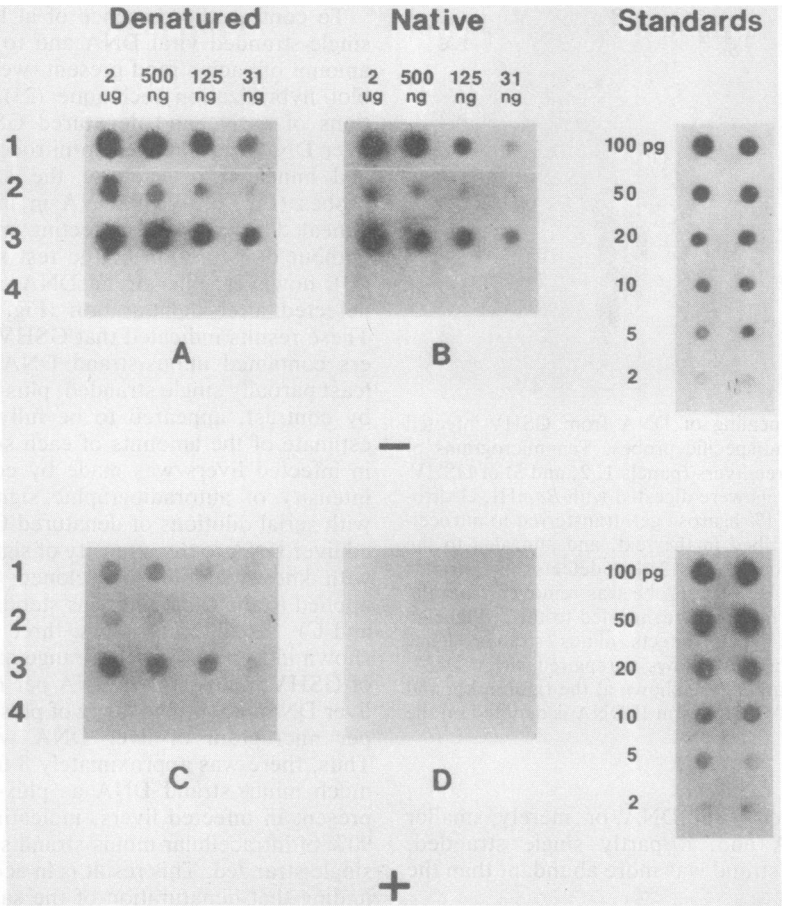

FIG. 6. Demonstration that minus-strand GSHV DNA in infected livers is partially single stranded and in excess of plus-strand DNA. Serial dilutions of whole cell DNA extracted from the livers of three infected squirrels (lanes 1 through 3 in each panel) and from the liver of one uninfected squirrel (lane 4 in each panel) were applied to two nitrocellulose filters with (A and C) and without (B and D) prior denaturation. Serial dilutions of cloned denatured GSHV DNA were applied to each filter as standards. Filters were then hybridized to probes which detect minus-strand (upper panel) or plus-strand (lower panel) DNA.

the amount of plus-strand DNA present at all S1 nuclease concentrations, again suggesting that in infected livers only a small proportion of the minus-strand DNA is paired with plus-strand DNA.

Covalent linkage of heterogeneous forms to protein. Like the virion DNA of HBV, GSHV virion DNA has a protein covalently linked to the minus strand (1). Protein-linked viral DNA species are extracted from aqueous solution with phenol; pretreatment of viral nucleic acid with proteases prevents this extraction. Using this assay, we determined which of the intracellular viral DNA forms are protein linked.

Liver DNA treated with proteinase K and then phenol extracted (Fig. 8, lanes 1 and 3) showed a large amount of heterogeneous DNA, as previously demonstrated (Fig. 2 and 5). However, when DNA was prepared from the same liver samples by phenol extraction without prior proteinase K digestion (Fig. 8, lanes 2 and 4), the heterogeneous DNA was absent, leaving only form I and form II viral DNA. These results indicate that heterogeneous DNA is bound to protein that can be largely removed by proteinase K. A tight, presumably covalent association is implied by the resistance of the protein-DNA complex to treatment with 2% SDS and 2% β-mercaptoethanol at 60°C. The absence of protein linked to the open circular form of GSHV DNA suggests that it is derived by nicking of the supercoiled DNA rather than by completion of both strands of heterogeneous DNA. Figure 8 also shows heterogeneous DNA that migrated more slowly than did monomeric open circular DNA. We have observed these larger forms of

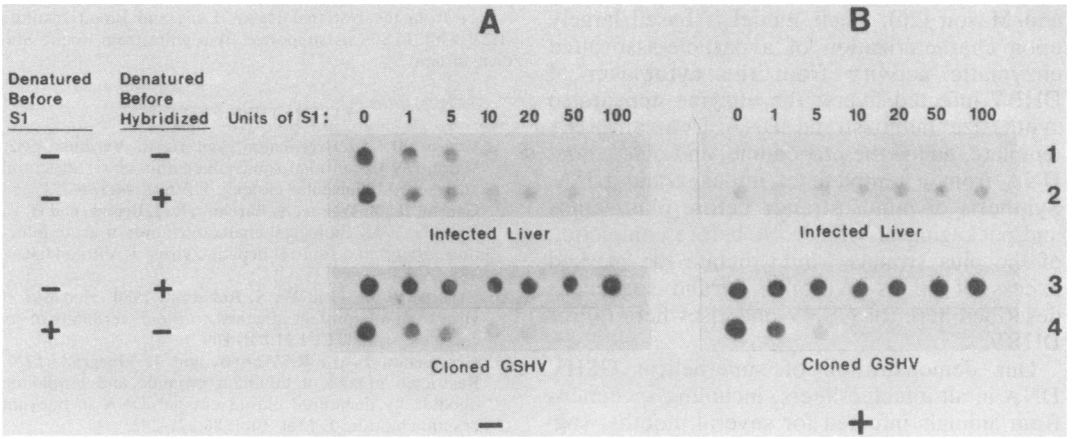

FIG. 7. Analysis of GSHV-infected liver DNA with S1 nuclease digestion and strand-specific probes. A total of 100 ng of whole cell DNA from the liver of an infected squirrel (lanes 1 and 2 in each panel) and control samples of 100 pg of cloned GSHV DNA (lanes 3 and 4 in each panel) were digested with increasing quantities of S1 nuclease as described in the text, applied to nitrocellulose filters by the dot blot technique as described in the text, and annealed to probes detecting minus-strand (A) or plus-strand (B) DNA. Lane 1 in (A and B), Liver DNA applied to the filter without denaturation of the sample. Lane 2 in (A and B), Liver DNA digested with S1 nuclease and denatured before application to the filter. Lane 3 in (A and B), Control samples of cloned GSHV DNA digested with S1 nuclease and denatured before application to the filter. Lane 4 in (A and B), Control samples of cloned GSHV DNA denatured before digestion with S1 nuclease.

GSHV DNA only in samples obtained from squirrels that had been infected for more than 1 year and have not yet elucidated their structure.

DISCUSSION

We have shown in this report that livers from GSHV-infected ground squirrels contain a variety of DNA forms that are undetectable within mature virions and are thus candidates for intermediates in viral replication. These forms include unit-length closed circular DNA and heterogeneous molecules that are protein linked, predominantly single stranded, and asymmetric, with minus-strand DNA in an 8- to 10-fold mass excess over plus-strand DNA. These findings for GSHV are compatible with recent results from other laboratories for HBV, woodchuck hepatitis virus, and DHBV: supercoiled circular viral DNA has been described in the livers of HBV-infected chimpanzees (16), DHBV-infected ducks, and woodchuck hepatitis virus-infected woodchucks (J. Summers and W. Mason, personal communication) and heterogeneous, asymmetrical protein-linked forms of viral DNA have been found in the livers of DHBV-infected ducks (9, 12).

These collective results can be interpreted in light of the hypothesis that replication of hepatitis B virus genomes proceeds through an RNA intermediate, as recently proposed by Summers

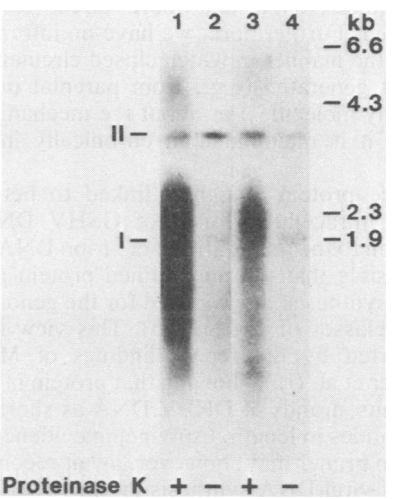

FIG. 8. Heterogeneous DNA linked to protein. DNA was prepared from two GSHV-infected livers by extraction with phenol, with and without prior proteinase K digestion, as described in the text. Ten micrograms of DNA was digested with *Bam*HI, electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and annealed to a ^{32}P-labeled $GSHV_{rep}$ probe as described in the text. Each set of two lanes shows the results for DNA from a single liver with (lanes 1 and 3) and without (lanes 2 and 4) proteinase K digestion. Size markers (in kb) are shown on the right; the positions of GSHV form I and form II DNA are indicated on the left.

and Mason (20). Their model is based largely upon characterization of a particle-associated enzymatic activity from the cytoplasm of DHBV-infected livers; the enzyme appears to synthesize minus-strand DNA from an RNA template, called the pregenome, and plus-strand DNA from a template of minus-strand DNA. Synthesis of minus strands before plus stands and packaging of viral DNA before completion of the plus strands would dictate the marked excess of minus over plus strand sequences described here for GSHV and elsewhere (9) for DHBV.

Our demonstration of superhelical GSHV DNA in all infected livers, including specimens from animals infected for several months, suggests that this form of viral DNA has some functional role in the replicative cycle. One attractive possibility, compatible with the Summers-Mason model (20), is that the superhelical DNA serves as a template for synthesis of pregenome RNA. Evidence favoring a nuclear location for closed circular forms of DHBV and woodchuck hepatitis virus DNA (J. Summers and W. Mason, personal communication) may support this possibility, since the synthesis of viral RNA is likely to be dependent upon a host RNA polymerase. However, nothing is known about the structure and site of synthesis of pregenome RNA, and more direct tests are required. Furthermore, we have no information about the manner in which closed circular DNA is first generated (e.g., from parental or from progeny molecules) or about the mechanism by which it is maintained in chronically infected cells.

Since protein is tightly linked to heterogeneous intracellular forms of GSHV DNA, in addition to the minus strand of virion DNA (1), it is possible that the unidentified protein primes DNA synthesis, as proposed for the genomes of other classes of viruses (25). This view is best supported by the recent findings of Molnar-Kimber et al. (12), showing that protein is linked to minus strands of DHBV DNA as short as 30 nucleotides in length. Convincing evidence for a protein primer may, however, await reconstruction of viral DNA synthesis in vitro.

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