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Virion DNA of Ground Squirrel Hepatitis Virus: Structural Analysis and Molecular Cloning

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The structure of the encapsidated DNA genome of ground squirrel hepatitis virus (GSHV) has been examined by restriction endonuclease cleavage, nucleic acid hybridization, and molecular cloning. GSHV virion DNA is a relaxed circular molecule of approximately 3,200 bases in length; most molecules harbor an extensive single-stranded region which is largely confined to one-half of the genome. The full-length viral DNA strand is covalently bound to protein. The single-stranded region can be repaired in vitro by the action of the endogenous virion polymerase, exogenously added DNA polymerase from avian myeloblastosis virus, or both. Restriction enzyme cleavage of viral DNA from different isolates demonstrated that multiple variants of GSHV exist in nature. The genomes of two such strains have been cloned in *Escherichia coli*, and their physical maps have been determined. Nucleic acid hybridization studies revealed that the strains share sequence homology with the DNA of human hepatitis B virus. Regions homologous to the coding regions for the surface and core antigens of human hepatitis B virus have been localized on the GSHV chromosome. Molecular cloning experiments have also led to the identification of a region of the viral genome which is altered in a procaryotic host.

Ground squirrel hepatitis virus (GSHV) is a small DNA virus first isolated from the blood of chronically viremic animals by Marion and coworkers (13). These investigators have demonstrated that the virus bears close morphological (13) and biochemical (7, 17) resemblance to the hepatitis B virus (HBV) of humans. Both are surrounded by an outer protein coat comprised primarily of a single polypeptide, termed surface antigen; the surface antigens of the two agents share a number of common tryptic peptides and are weakly cross-reactive in immunological tests (4, 7). The inner core of GSHV, like HBV, was shown to harbor a 3,200-base pair (bp) relaxed circular DNA molecule whose circularity is maintained by 5'-cohesive termini of about 300 bp in length (17). The molecules found in the virions have an extensive single-stranded region of fixed polarity, whose extent varies from molecule to molecule. Associated with this structure is a viral DNA polymerase, which is capable of repairing this gapped region in vitro to generate fully double-stranded relaxed circular genomes (13, 17). Siddiqui et al. (17) have recently cloned GSHV virion DNA in *Escherichia coli* and presented a physical map of their isolate.

In this paper, we further characterize the properties of GSHV DNA, as purified from the serum of infected animals. Virion DNA of GSHV, like its HBV counterpart (8), contains protein covalently linked to its intact (long) strand. We also demonstrate by restriction endonuclease cleavage analyses that multiple variants of this agent exist, even among animals residing in the same field. Molecular cloning experiments confirm these differences and have allowed construction of physical maps of the genomes of two strains. In addition, GSHV is shown to harbor a small genomic region which apparently undergoes alteration in a procaryotic host.

MATERIALS AND METHODS

Virus stocks. Sera were obtained by heart puncture of anesthetized animals and tested for hepatitis B surface antigen (HBsAg) cross-reactivity by solid-phase radioimmunoassay (6); positive sera were stored at −70°C until ready for use. All experiments were done on virions purified from the sera of single animals; sera were never pooled.

Enzymatic repair of viral DNA. The preparation of viral pellets and performance of the endogenous polymerase reaction are described in the accompanying paper (6). In our hands, the endogenous polymerase reaction rarely resulted in complete repair of the single-stranded region of the genome. To complete the

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repair, phenol-extracted viral DNA was incubated in an avian myeloblastosis virus (AMV) polymerase reaction in the presence of unlabeled deoxynucleoside triphosphates, exactly as described by Summers et al. (19).

Restriction endonuclease cleavage. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, Mass.), or BRL, Inc. (Bethesda, Md.), and reactions were conducted as recommended by the vendor.

Gel electrophoresis, DNA transfer, and hybridization. DNA samples in 40 mM Tris (pH 8)–5 mM NaAc–1 mM EDTA (1× TAE buffer) were made 10% in sucrose and loaded into the wells of horizontal agarose slab gels, cast in 1× TAE buffer. Gels were generally run for 6 to 9 h at 25 mA and room temperature. DNA bands were visualized by staining for 20 to 40 min with 1 μg of ethidium bromide per ml, followed by examination under a UV light source (UV Products, San Gabriel, Calif.).

For DNA transfer experiments, DNA in gels was denatured, neutralized, and transferred to nitrocellulose sheets as described by Southern (18). The filters were then baked for 2 h at 80°C in vacuo.

For DNA hybridization under stringent conditions, filters were preincubated at 41°C in 50% formamide–3× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate)–0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid) (pH 7)–200 μg of salmon sperm DNA per ml–150 μg of yeast RNA per ml–1× Denhardt solution. After 30 to 120 min, 1×10^6 to 1.5×10^6 cpm of ^{32}P-labeled DNA (generally 5 to 10×10^7 cpm/μg) was added to each filter in the same solution. After hybridization, filters were washed with 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) for 2 h at 50°C and then air dried and autoradiographed.

Low-stringency hybridization was carried out as above, except that the formamide concentration in the annealing mix was reduced to 25%. Filters were washed in 4× SSC–0.1% SDS for 2 to 3 h at 50°C.

Preparation of ^{32}P-labeled DNA. Cloned DNA fragments were radiolabeled in vitro by incubation with [^{32}P]dCTP, unlabeled dATP, dGTP, and dTTP, random primers of calf thymus DNA, and AMV polymerase, as previously described (16). The labeled product DNA was separated from unincorporated isotope by gel filtration over a column of Sephadex G-50. Column fractions were monitored by Cerenkov counting, and the void volume was pooled and concentrated by precipitation with 2 volumes of ethanol at −20°C.

HBsAg-specific probe was prepared by radiolabeling a 700-bp fragment of cloned HBV DNA extending from its unique *Xba*I site to its unique *Hpa*I site (20). Hepatitis B core antigen (HBcAg)-specific probe was prepared by radiolabeling the purified 400-bp *Bgl*II fragment of cloned HBV DNA (20).

Molecular cloning. For cloning in bacteriophage, the vector λgtWES-λB was used (12). The central region of the vector chromosome was excised by *Eco*RI digestion, and the flanking "arms" were purified by sucrose gradient sedimentation. For cloning, approximately 20 ng of *Eco*RI-digested ^{32}P-labeled GSHV virion DNA was annealed and ligated to 2.0 μg of λ arms. One-half of the ligation mix was then packaged into λ phage heads in vitro as previously described (1), and the resulting stock was plated on lawns of *E. coli* strain DP50 *supF* in 150-mm petri dishes. Recombinant phage were identified by plaque hybridization (2) with ^{32}P-labeled HBV DNA under low-stringency conditions, as defined above. Where indicated, isolates were plaque purified and rescreened several times in this fashion.

For cloning directly from virion DNA into plasmid vectors, 10 ng of ^{32}P-labeled strain 36 DNA was cleaved with *Eco*RI, phenol extracted, and then annealed and ligated for 18 h at 4°C to 100 ng of *Eco*RI-linearized pBR328 DNA which had been pretreated with alkaline phosphatase. The ligation products were introduced into *E. coli* strain HB101 by $CaCl_2$-mediated DNA transformation, and the entire transformation mix was plated on Luria-Bertani (LB) medium agar containing 50 μg of ampicillin per ml. Transformants were then screened for chloramphenicol sensitivity on LB medium agar with 25 μg of chloramphenicol per ml.

RESULTS

Characterization of virion DNA. As described in the preceding paper (6), sera obtained from two naturally infected animals (numbers 27 and 36) were used to transmit infection by inoculation to a group of captive ground squirrels. When recipients developed high circulating titers of surface antigen, they were bled and virions were pelleted from serum as described in Materials and Methods. Virus stocks obtained from animals infected with serum 27 were studied separately from stocks derived from serum 36.

To determine whether GSHV DNA shared sequence complementarity with HBV DNA and to assess the configuration of GSHV virion DNA before in vitro polymerase repair, GSHV DNA was extracted directly from a viral pellet after disruption of the virion with SDS and proteinase K. The viral DNA was electrophoresed through a 1% agarose gel, transferred to nitrocellulose paper (18), and annealed to cloned ^{32}P-labeled HBV DNA, under conditions of reduced hybridization stringency (Fig. 1A). Unrepaired GSHV DNA (lane b) is seen to be quite heterogeneous and migrates even more rapidly than unrepaired HBV DNA (lane a). As for HBV, no GSHV DNA is observed in the region of the gel where fully duplex, relaxed circular molecules would be expected to migrate. Similar results have been obtained with DNA from all four ground squirrel sera studied in this fashion (data not shown). This is in contrast to the situation which obtains for the recently described duck hepatitis B virus, in which a significant proportion of virions harbor fully duplex genomes (W. Mason, J. Taylor, G. Seal, and J. Summers, *in* H. Alter, J. Maynard, and W. Szmuness, ed., *Proceedings of the 1981 Symposium on Viral Hepatitis*, in press).

Figure 1B shows the results obtained when GSHV DNA is repaired in vitro. Virions from animals infected with sera 27 (lane a) and 36

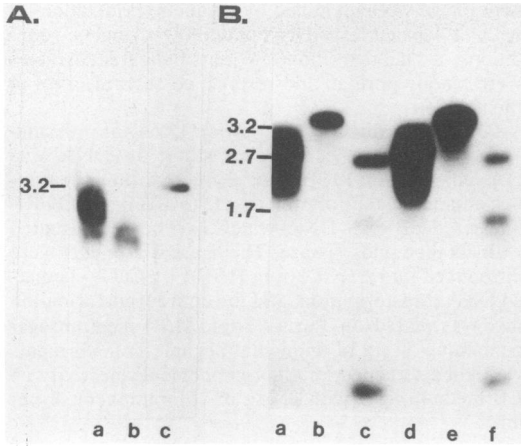

FIG. 1. Hybridization of unlabeled HBV and GSHV virion DNA with ^{32}P-labeled HBV DNA (A) and radiolabeling and *Pst*I cleavage of GSHV virion DNA (B). (A) Virions concentrated from 0.2 ml of infected human or ground squirrel serum were treated with 1% SDS and 1 mg of proteinase K per ml, and viral DNA was extracted as in the text. Ten percent of each sample was electrophoresed through 1% agarose, transferred to nitrocellulose paper after denaturation, and hybridized to 10^6 cpm of ^{32}P-labeled HBV DNA. Lane a, HBV DNA; lane b, GSHV DNA from animal PO33; lane c, 50 pg of *Eco*RI-cleaved plasmid pEC63 DNA. pEC63 is a recombinant plasmid pBR325 derivative harboring the 3.2-kb HBV genome cloned at its unique *Eco*RI site. (B) Virions of strain 27 (lanes a to c) or strain 36 (lanes d to f) were labeled with [^{32}P]dCTP in an endogenous polymerase reaction, product DNA was extracted as in the text, and aliquots were electrophoresed through 1% agarose gels (lanes a and d). The remainder of each DNA sample was repaired with AMV polymerase and unlabeled triphosphates and similarly electrophoresed (lanes b and e). Aliquots of AMV-repaired DNA digested with *Pst*I were electrophoresed in parallel (lanes c and f). After electrophoresis, the gel was dried and autoradiographed for 12 h at −70°C with an intensifying screen. In this figure and others, the numbers alongside the autoradiograms refer to the sizes (in kilobases) of linear forms of marker DNAs included in the experiments.

(lane d) were incubated in an endogenous polymerase reaction with [^{32}P]dCTP, and viral DNA was extracted and electrophoresed. In these cases, the repair reaction did not appear to proceed to completion, but these partially repaired forms could be fully repaired by subsequent incubation with AMV reverse transcriptase and excess unlabeled deoxynucleoside triphosphates (lanes b and e). This reveals that repaired molecules derived from both isolates are similar in size (3.2 kilobases [kb]). Cleavage of either preparation with *Pst*I (lanes c and f) reveals an identical set of labeled products, with major products of 2.7 and 0.5 kb in length and minor bands at 3.2 and approximately 1.7 kb. These restriction products will be considered more fully below.

We next sought to determine whether encapsidated GSHV DNA, like HBV DNA, is covalently linked to protein. As previously described for HBV (8), phenol extraction of GSHV virions results in the loss of viral DNA from the aqueous phase, unless the virions are first digested with proteolytic enzymes (Fig. 2). Virions pelleted

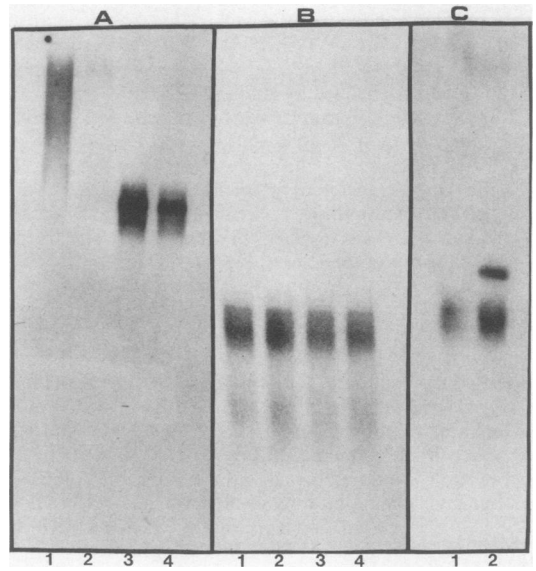

FIG. 2. Protein covalently linked to GSHV DNA. (A) Virions were pelleted from 0.2 ml of serum from an animal infected with a strain 27 GSHV isolate, and a brief endogenous polymerase reaction with [^{32}P]dCTP was performed as in the text. Virions were disrupted with 0.5% SDS–5% mercaptethanol at 55°C for 30 min. Ten percent of the sample was used for each analysis. Aliquots were electrophoresed without further treatment (lane 1), after phenol extraction (lane 2), after proteinase K treatment (lane 3), and after proteinase K treatment followed by phenol extraction (lane 4). Autoradiograms were prepared after electrophoresis as in the legend to Fig. 1. (B) Aliquots of [^{32}P]DNA-protein complex prepared as in (A) were first boiled for 45 s, quick-chilled on ice, and then electrophoresed without treatment (lane 1), after phenol extraction (lane 2), after proteinase K digestion (lane 3), or after proteinase K digestion followed by phenol extraction (lane 4). (C) Virions pelleted from 0.1 ml of serum from a GSHV-infected animal were disrupted with 0.5% SDS–5% β-mercaptoethanol without prior repair of the viral DNA. Aliquots (20% of the total sample) were used for each of the following analyses. Aliquots were electrophoresed after boiling for 45 s followed by phenol extraction (lane 1) or after proteinase K digestion followed by boiling and phenol extraction (lane 2). After electrophoresis, DNA was transferred to nitrocellulose sheets and annealed to cloned ^{32}P-labeled GSHV DNA under stringent conditions, and the filters were autoradiographed as in the legend to Fig. 1B.

from serum and radiolabeled in vitro in an incomplete endogenous polymerase reaction were examined in the phenol extraction assay. Before treatment with proteinase K, DNA extracted from virions lysed with 1% SDS migrates slowly and heterogeneously in a 1% agarose gel (Fig. 2A, lane 1); after phenol extraction, no ^{32}P-labeled GSHV DNA remains in the aqueous phase (Fig. 2A, lane 2). When the lysed virions are first incubated with proteinase K, the viral DNA is seen to migrate more rapidly in the gel (Fig. 2A, lane 3), and it remains in the aqueous phase after phenol extraction (Fig. 2A, lane 4). Thus, protein(s) bound to GSHV DNA can result in its extraction from the aqueous phase. To determine which strand of the viral DNA was associated with protein, the radiolabeled viral DNA was denatured by boiling (in 1% SDS) before phenol extraction. This dissociates the short (S) strand, which has been elongated with radiolabeled nucleotides, from the long (L) strand, which remains unlabeled. The mobility of the radiolabeled S strand is the same irrespective of proteinase K digestion or phenol extraction (Fig. 2B). This strongly suggests that protein is associated with the long L strand, although it does not rule out the possibility that S-strand-bound protein is removed by boiling in SDS. To further define the site of protein attachment, we examined unrepaired DNA extracted directly from virions. Unlabeled GSHV virions were lysed with 1% SDS, and their DNA was denatured by boiling and then phenol extracted; DNA remaining in the aqueous phase was electrophoresed through 1% agarose, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled GSHV DNA. Figure 2C (lane 1) shows that, as before, only the heterogeneous (S) strands remain in the aqueous phase. However, if the preparation is protease treated before phenol extraction, an additional discrete band representing the viral L strand can be observed (Fig. 2C, lane 2). This indicates that the protein is attached to the L strand and that the DNA-protein bond is stable to boiling in SDS and mercaptoethanol.

Multiple strains of GSHV. When virion DNA preparations radiolabeled as in Fig. 1B were cleaved with the enzyme *Eco*RI, clear differences in the sizes of the major products were noted between stocks derived from serum 27 and those derived from serum 36 (Fig. 3). Before cleavage, all samples appear similar in size (lanes a, c, and e), as noted above. Cleavage of viral DNA from a recipient of serum 27 (lane b) reveals the major product to be a 3.2-kb linear species, indicating that the parental population is composed chiefly of molecules harboring a single *Eco*RI recognition site. In contrast, *Eco*RI cleavage of viral DNA from two animals infected with serum 36 shows the major radiolabeled product to be a single band of approximately 1.5 kb. This indicates that most molecules in these sera harbor at least two *Eco*RI sites. Since radiolabel is only incorporated into the (repaired) single-stranded region, fragments derived predominantly from the duplex portion of the unrepaired molecule are underlabeled in this analysis and may not be detected. (In both cases, minor radiolabeled products are again visible [discussed further below].) We have subsequently examined sera from three individuals inoculated with serum 27 and from three recipients of serum 36; in all cases, there was complete concordance of *Eco*RI restriction pattern with the lineage of the isolate (data not shown). Serial inoculation experiments described in the preceding paper demonstrated that viral infection could be further transmitted to a second cohort of susceptible recipients (6). When virions from such recipients were similarly examined, the respective *Eco*RI cleavage patterns were again preserved (M. A. Schofield and D. Ganem, unpublished data). Thus, these restriction pattern differences behave as stable genetic

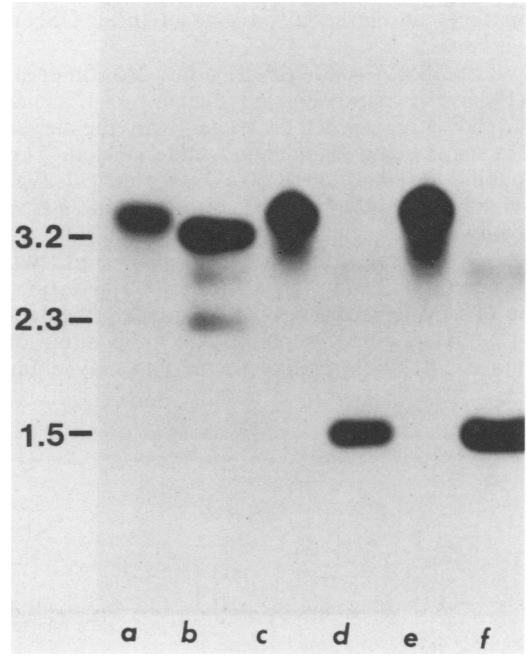

FIG. 3. *Eco*RI cleavage analysis of GSHV DNA of strains 27 and 36. Virion DNA was extracted from the sera of animals infected with serum 27 (lanes a and b) or 36 (lanes c to f) and radiolabeled and repaired as in the legend to Fig. 1B. The repaired DNAs (lanes a, c, and e) were cleaved with *Eco*RI (lanes b, d, and f, respectively) and electrophoresed through 1% agarose gels, which were then autoradiographed as in the legend to Fig. 1.

markers during serial passage of these GSHV variants.

The GSHV isolate described by Marion et al. (13) gives two radiolabeled fragments of 1.75 and 1.5 kb after *Eco*RI cleavage, with the larger fragment being significantly underlabeled. The published restriction map of this isolate (17; Fig. 4) differs slightly from that of our strain 36 (see below). We have trapped a single naturally infected animal whose virions displayed two bands of 1.75 and 1.5 kb after *Eco*RI digestion; in this instance, however, the smaller fragment was underlabeled, implying that the position of the *Eco*RI sites relative to the gap may differ between these two isolates. We have not further characterized this isolate.

Molecular cloning of GSHV genomes. Examination of the restriction patterns of virion DNAs presented in Fig. 1B and 3 reveals the frequent presence of minor products. For instance, *Eco*RI cleavage of DNA from one strain 27 stock produces minor fragments of 2.8 and 2.3 kb in length, in addition to the major 3.2-kb linear product (Fig. 3). Similarly, complete *Eco*RI cleavage of DNA from sera of several (but not all) strain 36 recipients reveals minor products of 3.2 and 2.9 kb (Fig. 3). To further characterize the strain differences noted above and to deter-

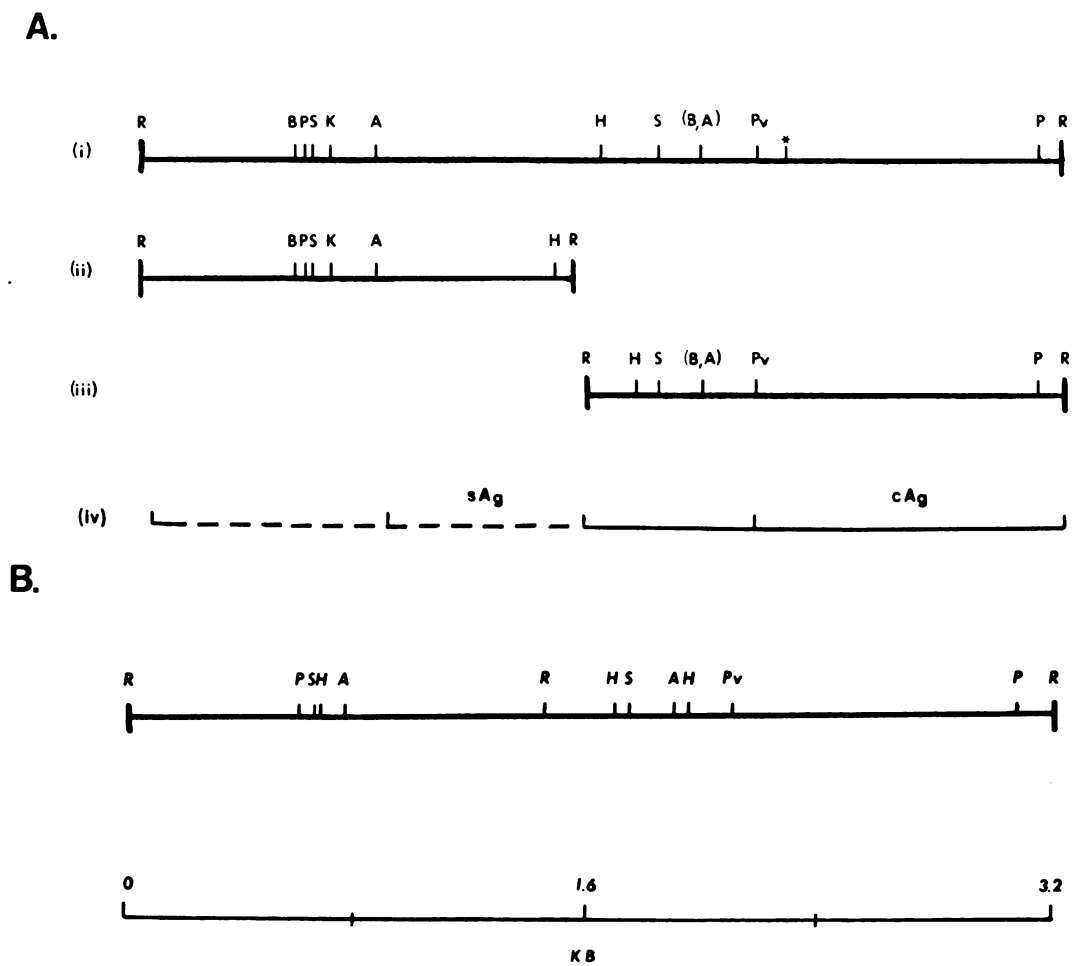

FIG. 4. Restriction maps of cloned GSHV isolates. (A) Restriction map of the 3.2-kb strain 27 GSHV insert (line i), of the 1.5-kb strain 36 subgenomic *Eco*RI fragment (line ii), and of the 1.65-kb strain 36 subgenomic *Eco*RI fragment (line iii). Line iv depicts the locations of the GSHV DNA sequences homologous to the coding regions for HBsAg and HBcAg. The approximate position of the single-stranded region is indicated by the dashed line. (B) Restriction map of the GSHV isolate cloned by Siddiqui et al. (17). Abbreviations: A, *Ava*I; B, *Bst*EII; H, *Hin*cII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; S, *Sac*I. *Position of the *Cla*I site in strain 27 virion DNA. [This site is not represented in the clones shown in (A).]

mine the origin of the frequently observed minor restriction products, molecular cloning of GSHV genomes was undertaken with DNA purified from the serum of one animal infected with strain 36 and one animal infected with strain 27. Radiolabeled, repaired DNA of strain 27 (prepared from the serum of animal P015) was cleaved with *Eco*RI, and the limit products (Fig. 5A, lane b) were cloned into the *Eco*RI site of λgtWES-λB. Ten isolates were plaque purified and analyzed for GSHV inserts; all 10 proved to harbor full-length (3.2-kb) GSHV genomes. Extensive characterization of three such inserts showed them to be indistinguishable, and one was then subcloned into the *Eco*RI site of plasmid pBR328 (Fig. 6B, lane a).

For strain 36 cloning, repaired virion DNA from animal P052 was cleaved with *Eco*RI. One-half of the sample was cloned into the *Eco*RI site of pBR328; the remainder was cloned into the *Eco*RI site of λgtWES-λB (see Materials and Methods). Three pBR328 clones were positive for GSHV DNA sequences; two harbored inserts of 1.5 kb, and one contained a 1.65-kb insert (Fig. 6A). (The two 1.5-kb inserts proved to be identical by restriction mapping.) Interestingly, no inserts of 1.5 or 1.65 kb were recovered in λgtWES. Six of the 210 GSHV-positive plaques were screened, and all six harbored 2.9-kb inserts of GSHV DNA (Fig. 6B); the origin of the 2.9-kb inserts is considered below (see Discussion). We presume that the absence of 1.5- and 1.65-kb inserts from this small collection reflects inefficient packaging or propagation of inserts of this size in the λgtWES vector.

Fine structure of cloned GSHV DNA. The full-length GSHV clone from strain 27 and the 1.65- and 1.5-kb inserts from strain 36 have been physically mapped with a variety of restriction endonucleases. The maps of these three cloned DNAs are shown in Fig. 4A, aligned to emphasize regions of similarity. Fortunately, the obvious homologies of recognition sites between the two strains allows the correct orientation of the subgenomic fragments of strain 36. In Figure 4B, these maps are compared with the map of a GSHV clone independently derived by Siddiqui et al. (17). Again, although the maps display overall similarity, clear differences exist. For example, two regions (map positions 0.65 and 2.2) of the clone of Siddiqui et al. harbor *Hin*cII sites not present in either strain 27 or strain 36.

Radiolabeled GSHV DNA prepared from the 3.2-kb GSHV clone from strain 27 has been used to further investigate the nature of the DNA in strain 36 virions. Unlabeled, repaired strain 36 virion DNA was digested with *Eco*RI; the cleavage products were electrophoresed as before and annealed to cloned ^{32}P-labeled GSHV DNA, yielding the expected two bands of 1.65 and 1.5 kb (Schofield and Ganem, unpublished data). That only the 1.5-kb fragment is radiolabeled in the endogenous polymerase reaction (Fig. 3) localizes the single-stranded region predominantly to this portion of the molecule. Cleavage of radiolabeled genomes with *Pst*I (Fig. 1B), *Bst*EII, or *Ava*I (data not shown) is also consistent with this assignment. However, since repair of this region by the endogenous polymerase was generally incomplete (see above), extension of the gap region 0.2 to 0.3 kb beyond the central *Eco*RI site of Fig. 4A is not excluded. The position of the cohesive 5′ termini on the genome (see below) makes it likely that this is indeed the case.

Previous work in several laboratories has localized the coding regions for the surface and core antigens of HBV on the physical map of the cloned HBV genome (5, 15, 20). We have used this information to locate the homologous regions on the GSHV map. This was done by preparing radiolabeled HBV probes specific to the HBsAg and HBcAg coding regions (see above) and annealing these DNAs to subgenomic fragments of the strain 36 GSHV clones. All HBsAg coding region homology was localized to a 700-bp *Ava*I-*Eco*RI fragment (Fig. 4A). Thus, as for HBV, the putative surface antigen coding region falls within the single-stranded portion of the genome. Homology with the HBcAg probe was similarly determined to be restricted to the 1.05-kb *Pvu*II-*Eco*RI fragment (Fig. 4A), but the paucity of restriction sites in this region of the map precluded more precise localization. While this work was in progress, similar results were reported by Siddiqui et al. (17).

Marion et al. (13) have previously shown that circular GSHV DNA can be reversibly converted to a linear form by gentle heat treatment, implying the presence of cohesive termini as previously noted in HBV DNA (16a). We have mapped the location of this site (in strain 27) to the region of the unique *Pvu*II site by cleavage of the linear molecules so generated with a variety of restriction enzymes (data not shown). Siddiqui et al. (17) have demonstrated that the cohesive ends of the genome are similarly located in their GSHV isolate.

Region of GSHV DNA which is unstable in procaryotic hosts. When virion DNA from the serum of a strain 27 carrier is radiolabeled in an endogenous reaction and cleaved with the enzyme *Cla*I, all molecules are cleaved at a unique site to generate 3.2-kb linear molecules. Digestion with both *Cla*I and *Eco*RI yields a major radiolabeled product of 2.3 kb (Fig. 5A). However, during restriction mapping of the cloned strain 27 isolate, we noted that the expected *Cla*I recognition site was not present in the cloned

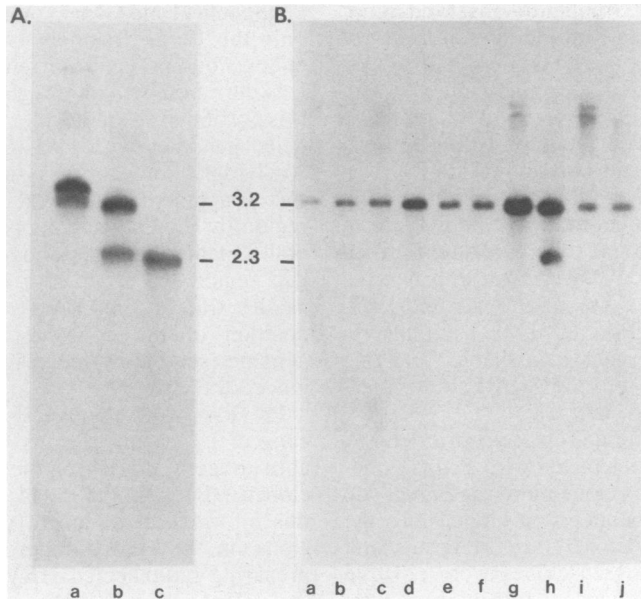

FIG. 5. Detection of GSHV recombinants harboring the *Cla*I recognition site. (A) Virion DNA from animal P015 (bearing strain 27) was extracted and radiolabeled as in the legend to Fig. 1B. A total of 10^4 trichloroacetic acid-precipitable cpm of this material were electrophoresed through 1.4% agarose without further treatment (lane a), after cleavage with *Eco*RI (lane b), or after cleavage with *Eco*RI and *Cla*I (lane c). Gels were autoradiographed as in the legend to Fig. 1B. (B) Stocks of λgtWES-GSHV recombinants derived from the cloning of the material shown in (A) (lane b) were prepared from the initial recombinant plaques without further plaque purification. DNA (0.2 μg per lane) from five such stocks was cleaved with either *Eco*RI alone (lanes a, c, e, g, and i) or *Eco*RI and *Cla*I (lanes b, d, f, h, and j). The products were electrophoresed through 1.4% agarose gels, transferred to nitrocellulose paper, and annealed with 10^6 cpm of ^{32}P-labeled GSHV DNA. Fragments of 2.3 kb were detectable in trace amounts in lanes b and d in the original autoradiogram, but the bands are difficult to visualize after photographic reproduction.

DNA. Examination of several other plaque-purified isolates from the same cloning experiment likewise demonstrated the absence of this site. However, when the initial isolates were examined before plaque purification, different results were obtained. Phage DNA prepared directly from these isolates was digested with *Eco*RI to excise the 3.2-kb GSHV insert. A portion of each preparation was then exposed to *Cla*I, and the limit digestion products of both reactions were electrophoresed in parallel through 1% agarose gels. GSHV-specific sequences were identified by annealing with cloned ^{32}P-labeled GSHV DNA; the results of this experiment are shown in Fig. 5B. Of eight isolates so examined (of which five are shown in Fig. 5B), five revealed variable amounts of the expected 2.3-kb fragment, in addition to the major *Cla*I-resistant species, indicating that some molecules retaining the *Cla*I site were still present at this level of passage. (The other [0.8-kb] *Eco*RI/*Cla*I product was also visualized in stocks harboring higher quantities of *Cla*I-sensitive species.) Two separate attempts to subclone such molecules into pBR328 from a stock in which almost 40% of the molecules were *Cla*I sensitive yielded only *Cla*I-resistant species in 25 of 25 independent subclones (Schofield and Ganem, unpublished data).

Like the strain 27 clones just described, all clones derived from strain 36 also lack recognition sites for *Cla*I (Fig. 4A) despite the presence of this site in the starting material. The position of the *Cla*I site in strain 27 has been approximately localized by restriction cleavage analysis of a λ-GSHV isolate harboring both GSHV species; this site is denoted by the asterisk in Fig. 4 (line i).

We presume that the absence of the *Cla*I site in our most purified stocks reflects the loss or alteration of this site during cloning, rather than sequence heterogeneity of the starting material, because of (i) repeated failures to obtain *Cla*I-sensitive molecules in individual clones, (ii) the high proportion of initial plaques harboring both species, and (iii) the absence of demonstrable *Cla*I-resistant species in the starting DNA purified from serum. The nature of the event result-

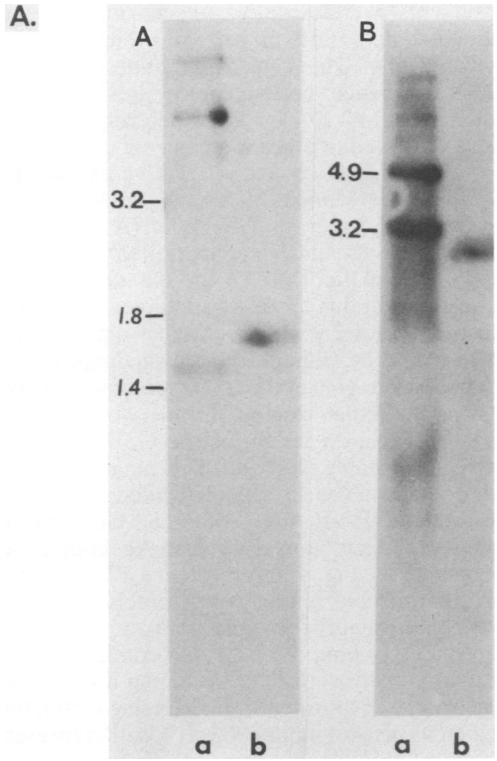

FIG. 6. Molecular clones isolated from GSHV DNA. (A) pBR328-containing recombinant plasmids (0.3 μg per lane) bearing inserts of 1.5 (lane a) and 1.65 (lane b) kb were cleaved with *Eco*RI, electrophoresed through 1.3% agarose gels, transferred to filters of nitrocellulose, and annealed to 10^6 cpm of ^{32}P-labeled HBV DNA under conditions of reduced hybridization stringency. Numbers in (A) and (B) indicate the length (in kilobases) of size markers included in a parallel lane. (B) pBR328-containing recombinant plasmid (pBA131) bearing the 3.2-kb GSHV insert from strain 27 (lane a) and a λgtWES recombinant phage bearing a 2.9-kb strain 36 insert (lane b) were cleaved with *Eco*RI, electrophoresed and transferred as in (A), and then annealed to 10^6 cpm of ^{32}P-labeled pBA131 DNA.

ing in the elimination or modification of the *Cla*I site is unknown (see below).

DISCUSSION

Genome structure of GSHV. In agreement with earlier reports by Robinson and co-workers (13, 17), the experiments presented here document that the encapsidated genome of GSHV resembles that of HBV in terms of (i) its overall configuration (partially duplex circular DNA of 3.2 kb, with an extensive single-stranded region; Fig. 1) and (ii) the disposition of its putative sAg and cAg coding regions relative to the gap (Fig. 5A). In addition, several further features of the GSHV genome are reported here. First, like HBV, GSHV harbors a protein covalently associated with the L strand of the viral DNA; this protein results in the extraction of viral DNA from aqueous solution by phenol. The site of attachment of this protein to the GSHV L strand remains to be defined. Our experiments do not exclude the possibility that protein(s) may also be attached to the S strand. However, such protein-S strand interactions, if present, either are unstable to boiling in SDS or do not affect the behavior of the DNA in phenol extraction.

Second, work presented in this and the preceding paper documents the existence of multiple variants of GSHV, first detected by their differing restriction endonuclease cleavage patterns. These cleavage differences are stable on serial propagation in animals and are faithfully reflected in the molecularly cloned genomes depicted in Fig. 4. No major biological differences between strains have yet been discerned (6). In these respects, the restriction site variants of GSHV resemble the described subtypes of HBV. However, we have not yet determined whether antigenic differences between these strains can be identified, as for the HBV subtypes.

Third, molecular cloning experiments have led to the generation of detailed restriction maps of two GSHV strains. With these maps, we have been able to localize two important topographical landmarks of the genome, the cohesive termini and the single-stranded gap. This information, in turn, has helped clarify the origins of many of the minor DNA products of restriction endonuclease cleavage of uncloned virion DNA.

Minor restriction products of virion DNA. Cleavage of radiolabeled virion DNA with restriction enzymes gives rise principally to the species predicted by the restriction maps of the cloned genomes (Fig. 1 and 3). However, numerous minor products are also observed. These minor products can be divided into three classes.

(i) The first (and usually most abundant) class consists of molecules which probably arise from disruption of the cohesive terminus before or after cleavage. Examples of such fragments include the 2.3-kb *Eco*RI fragment of strain 27 (Fig. 5A) and the 1.7-kb *Pst*I fragment of either strain (Fig. 1B). Several lines of evidence indicate that these fragments derive from disruption of the cohesive ends of the molecule. First, their sizes are consonant with this mechanism and the intensity of their radiolabeling corresponds to the proportion of the gap region they would be expected to harbor. Second, their relative amounts among the products are proportional to the amounts of linear molecules in the undigested stock (cf. for example, the relative amounts

of the 1.7-kb *Pst*I fragment in the two reactions of Fig. 1B, or the relative amounts of the 2.3-kb *Eco*RI fragment in Fig. 3 and 5A). Third, if the cohesive ends of the genome are deliberately heat denatured after cleavage, these fragments become the major products observed (unpublished data).

Another piece of evidence supporting this origin for these fragments is derived from molecular cloning. Restriction fragments derived by disruption of the cohesive ends should have heterologous (noncomplementary) termini; such molecules should not be readily cloned in standard cloning vectors. The *Eco*RI products of a strain 27 isolate are shown in Fig. 5A; in this stock, the minor (2.3-kb) *Eco*RI fragment represents over one-third of the product. However, in accord with expectation, none of the 12 GSHV-specific clones recovered from this stock in λgtWES were of this size.

We do not know how linear molecules are generated in stocks of virion DNA without application of denaturating conditions. It is possible that varying amounts of gapped linear molecules are encapsidated in vivo and that in vitro repair of these molecules gives rise to the corresponding radiolabeled linear duplexes. Alternatively, they could be derived from circular genomes by repair of the cohesive termini in vitro, in either the endogenous polymerase reaction or exogenous repair with AMV polymerase. Interestingly, Mason et al. (14) have noted that incubation with AMV polymerase can linearize relaxed circular genomes of the duck HBV.

(ii) A second class of minor restriction products probably results from incomplete repair of the gap region. An example of such a product would be the 3.2-kb fragment seen after cleavage of some strain 36 preparations with *Eco*RI (Fig. 3). A fragment of this size cannot result from disruption of the cohesive termini. The amounts of fragments of this class vary between preparations, depending on the degree of repair of the molecule. Because of this, we presume that they result from incomplete repair, which leaves one (or more) restriction site single stranded and thus inaccessible to cleavage with the appropriate enzyme. This phenomenon has also been noted in similar analyses of HBV DNA (11).

(iii) Infrequently, a third class of fragments is observed, for which we have as yet no clear explanation. The sizes of these fragments are such that they cannot be accounted for by either disruption at the cohesive ends of the genome or incomplete repair. Examples of this class include the 2.8-kb *Eco*RI fragment seen in some strain 27 preparations, the 2.9-kb fragment sometimes seen after *Eco*RI cleavage of strain 36 (Fig. 3), and the 2.9-kb *Eco* fragment repeatedly cloned from strain 36 DNA (Fig. 6B). Such fragments were usually present in very low amounts and only in certain preparations of virion DNA. They could, in principle, reflect DNA sequence heterogeneity present in the uncloned viral stocks; such heterogeneity has been observed in other systems, e.g., the human papovavirus BK (9). An alternative explanation for the 2.9-kb cloned DNA might be that it arose by rearrangement of GSHV DNA sequences during cloning, since examination of both virion and intrahepatic DNA from the animal from whose serum this clone was obtained has shown no product of 2.9 kb after *Eco*RI digestion (B. Weiser and D. Ganem, unpublished data).

A specific region of the GSHV genome is altered during molecular cloning. In this work we also have identified a region of the GSHV genome which is unstable in *E. coli*. This region (in strain 27) contains a *Cla* site and is located adjacent to the unique *Pvu*II site, within or close to sequences which anneal to HBcAg coding sequences (see Fig. 4 and reference 17). The size of this unstable region is not precisely known but is presumably less than 100 to 150 bp, since molecules lacking the *Cla*I site comigrate electrophoretically with those retaining it (Fig. 5B). The nature of the alteration occurring within this region is as yet unknown. It could represent chemical modification (e.g., methylation), point mutation, or a small sequence rearrangement (deletion/insertion); recent work in *E. coli* has demonstrated that regions flanked by direct repeats of base sequences are often highly unstable and undergo frequent spontaneous deletion (3).

The presence of this unstable region emphasizes the need for caution in the evaluation of eucaryotic sequences cloned in *E. coli*. The strain 27 clones we obtained appeared to be of wild-type size; only the fortuitous occurrence of a unique restriction site in the altered region allowed us to identify this change. In the last analysis, only the demonstration of biological activity is proof that a cloned sequence retains all of the essential features of the parental molecule. At present, although several clones of HBV and GSHV have been described (5, 15, 17, 20), proof of biological activity has not been presented for any cloned genome, including those reported here.

Regardless of their biological activity, however, the cloned genomes described here serve as useful reagents for the detection of virus-specific nucleic acid sequences in infected animals. Using these molecules as hybridization probes, we have demonstrated that GSHV, like its human counterpart, is strongly hepatotropic (6). Studies of the structure of intracellular viral DNA and RNA, using these reagents, are presently in progress in our laboratory.

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LITERATURE CITED

1. **Blattner, F., A. Blechl, K. Denniston-Thompson, H. Faber, J. Richards, J. Sightom, P. Tucker, and O. Smithies.** 1978. Cloning human fetal gamma globin and mouse alpha-type globin DNA: preparation and screening of shotgun preparation. Science **202**:1279–1284.
2. **DeLorbe, W., P. Luciw, H. M. Goodman, H. E. Varmus, and J. M. Bishop.** 1980. Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. J. Virol. **36**:50–61.
3. **Farabaugh, P., U. Schmeisser, M. Hofer, and J. H. Miller.** 1978. Genetic studies of *lac* repressor: on the molecular nature of spontaneous hotspots in the *lac* I gene of *E. coli*. J. Mol. Biol. **126**:847–863.
4. **Feitelson, M., P. Marion, and W. S. Robinson.** 1981. Antigenic and structural relationships of the surface antigens of hepatitis B virus, ground squirrel hepatitis virus, and woodchuck hepatitis virus. J. Virol. **39**:447–454.
5. **Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charney.** 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. Nature (London) **281**:646–650.
6. **Ganem, D., B. Weiser, A. Barchuk, R. J. Brown, and H. E. Varmus.** 1982. Biological characterization of acute infection with ground squirrel hepatitis virus. J. Virol. **44**:366–373.
7. **Gerlich, W., M. Feitelson, P. Marion, and W. Robinson.** 1980. Structural relationships between the surface antigens of ground squirrel hepatitis virus and human hepatitis B virus. J. Virol. **36**:787–795.
8. **Gerlich, W., and W. Robinson.** 1980. Hepatitis B virus contains protein attached to the 5′ terminus of its complete DNA strand. Cell **21**:801–809.
9. **Howley, P., M. Mullarkey, K. Takemoto, and M. A. Martin.** 1975. Characterization of human papovavirus BK DNA. J. Virol. **15**:173–181.
10. **Ish-Horowicz, D., and J. Burke.** 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2998.
11. **Landers, T., H. Greenberg, and W. S. Robinson.** 1977. The structure of hepatitis B virus DNA and nature of the endogenous DNA polymerase reaction. J. Virol. **23**:368–376.
12. **Leder, P., D. Tiemier, and L. Enquist.** 1977. EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: the gtWES system. Science **196**:175–177.
13. **Marion, P., L. Oshiro, D. Regnery, G. Scullard, and W. S. Robinson.** 1980. A virus in Beechey ground squirrels which is related to hepatitis B virus in man. Proc. Natl. Acad. Sci. U.S.A. **77**:2941–2945.
14. **Mason, W., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. **36**:829–836.
15. **Pasek, M., T. Goto, W. Gilbert, B. Zink, H. Schaller, P. MacKay, G. Leadbetter, and K. Murray.** 1979. Hepatitis B virus genes and their expression in *E. coli*. Nature (London) **281**:575–579.
16. **Payne, G. S., S. A. Courtneidge, L. B. Crittenden, A. M. Fadly, J. M. Bishop, and H. E. Varmus.** 1981. Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for the maintenance of the tumor state. Cell **23**:311–322.

16a. **Sattler, F., and W. S. Robinson.** 1979. Hepatitis B viral DNA molecules have cohesive ends. J. Virol. **32**:226–233.

17. **Siddiqui, A., P. Marion, and W. Robinson.** 1981. Ground squirrel hepatitis virus DNA: molecular cloning and comparison with hepatitis B virus DNA. J. Virol. **38**:393–397.
18. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
19. **Summers, J., J. Smolec, and R. Snyder.** 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. U.S.A. **75**:4533–4537.
20. **Valenzuela, P., P. Gray, M. Quiroga, J. Zaldivar, H. M. Goodman, and W. J. Rutter.** 1979. Nucleotide sequence of the gene coding for the major protein of hepatitis B virus surface antigen. Nature (London) **280**:815–819.