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Ground Squirrel Hepatitis Virus DNA: Molecular Cloning and Comparison with Hepatitis B Virus DNA

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Ground squirrel hepatitis virus (GSHV) shares many ultrastructural antigenic, molecular, and biological features with hepatitis B virus (HBV) of humans, indicating that they are members of the same virus group. Both viruses contain small circular DNA molecules which are partially single stranded. Here, we ligated an endonuclease *Eco*RI digest of GSHV DNA with *Eco*RI-cleaved plasmid vector pBR322 and cloned recombinant plasmids in *Escherichia coli* C600. Two cloned recombinants were characterized. One (pGS2) was found to contain only part of the GSHV genome, and the other (pGS11) was found to contain the entire viral DNA. A restriction endonuclease cleavage map of the GSHV insert in pGS11 and the locations of certain physical features of the virion DNA were determined. The relative positions of the single-stranded region, the unique 5′ end of the short DNA strand, and the unique nick in the long DNA strand in GSHV DNA were found to be the same as those previously described for HBV DNA. Hybridization with an HBV [^{32}P]DNA probe containing the apparent coding sequence for the major polypeptide of HBV surface antigen and a probe containing the putative coding sequence for the major polypeptide of the HBV core revealed specific homology with different restriction fragments of GSHV DNA. The two homologous regions had approximately the same locations relative to the single-stranded region, the 5′ end of the short strand, and the nick in the long strand in the two viral DNAs. These results suggest that in both viruses the genes for the major HBV surface antigen and core polypeptides have the same locations relative to unique physical features of the viral DNAs.

Viruses recently found in three animal species (woodchucks, Beechey ground squirrels, and domestic ducks) share ultrastructural, molecular, biological, and antigenic features of hepatitis B virus (HBV), suggesting that they are members of the same unique virus group (2, 6, 7, 17, 21). The viruses all appear to infect hepatocytes, they commonly cause persistent infection with high concentrations of morphologically similar incomplete as well as complete virus particles continually in the blood, and persistent infection, at least with HBV (19) and woodchuck hepatitis virus (17), is associated with primary liver carcinoma. The three mammalian viruses, HBV, woodchuck hepatitis virus, and ground squirrel hepatitis virus (GSHV), each appear to have characteristic virion surface, core, and e antigens, which cross-react with their counterparts. These viruses contain small circular DNA molecules which are partially single stranded (5–7, 11, 17, 18, 21). A DNA polymerase (DNA nucleotidyltransferase) activity in the virion core repairs the single-stranded region to make fully double-stranded molecules of approximately 3,200 base pairs (bp). The repair reaction is initiated at the 3′ end of the short DNA strand, the location of which varies in different molecules within a region consisting of 50% of the molecule. The reaction is terminated when the uniquely located 5′ end of this strand is reached. The long DNA strand contains a nick at a specific site, and that is approximately 300 bp from the 5′ end of the short strand in HBV (12, 13).

GSHV, first identified in this laboratory (6), represents an important model of viruses of this class. Although its surface, core, and e antigens partially cross-react with those of HBV (2, 6), the two major polypeptides of GSHV surface antigen are distinctly smaller in size than their counterparts in HBV, and only approximately one-third of the tryptic peptides of the major polypeptides of the surface antigens of the two viruses are identical, whereas two-thirds are different (2). Such studies provide one measure of the relatedness of these two viruses. To compare the structures of the two viral DNAs and to provide a further measure of the relatedness of the two viruses, we have cloned GSHV DNA in bacterial cells, constructed a map of physical features of the viral DNA, and identified regions

of GSHV DNA which are homologous to specific HBV DNA base sequence regions (including the HBV surface antigen coding region).

GSHV DNA has been found to be a circular molecule in electron micrographs (data not shown), and restriction endonuclease *Eco*RI cleaves the DNA at two sites to produce fragments of 1,750 (*Eco*RI-A) and 1,500 (*Eco*RI-B) (6). The *Eco*RI B fragment includes the entire single-stranded region of the viral DNA. To clone GSHV DNA, 50 ng of virion DNA made fully double stranded in a virion DNA polymerase reaction (12) was partially digested with *Eco*RI (New England Biolabs) to obtain some unit-length linear molecules as well as fragments of 1,750 and 1,500 bp. The partial digest was then ligated to *Eco*RI-cleaved, phosphatase-treated pBR322 plasmid vector (0.5 μg) (15). The ligation reaction mixture was used to transform *Escherichia coli* C600 (*hsdR hsdM*$^+$) in P3 + HV1 containment conditions. Recombinant clones detected by colony hybridization (3) were found to contain either the entire GSHV genome or only *Eco*RI-B inserted at the *Eco*RI site of the plasmid DNA. Hybrid plasmids, designated pGS2 and PGS11, isolated from two representative clones by equilibrium centrifugation in ethidium bromide-containing CsCl density gradients were examined by agarose gel electrophoresis after digestion with *Eco*RI or *Hin*dIII. Lanes 5 through 8 of Fig. 1 show DNA bands detected by ethidium bromide staining of the gel, and lanes 1 through 4 show the autoradiogram after transfer of the DNAs from the gel to a nitrocellulose filter and hybridization with a GSHV [^{32}P]DNA probe radiolabeled in a virion DNA polymerase reaction to identify fragments containing sequences homologous to the single-stranded region of GSHV DNA. *Hin*dIII digestion gave linear forms of pGS2 (5,800 bp) (lanes 1 and 8) and pGS11 (7,636 bp) (lanes 2 and 7), each larger than the linear form of the pBR322 vector (4,364 bp) (lane 9). It became clear upon *Eco*RI digestion that pGS2 (lanes 3 and 6) contained an insert consisting of only the 1,500-bp *Eco*RI fragment of GSHV DNA and that pGS11 (lanes 4 and 5) contained both the 1,750- and the 1,500-bp *Eco*RI fragments of GSHV DNA, which constitute the entire viral genome. Thus, pGS2 appears to contain only the *Eco*RI B fragment of GSHV DNA, and pGS11 appears to contain the entire GSHV DNA.

A map of restriction endonuclease cleavage sites for the GSHV DNA insert in pGS11 was determined by the method of Smith and Birnsteil (14), and the positions of the 5′ end of the short strand, the nick in the long strand, and the single-stranded region in virion DNA were determined as previously described for HBV DNA

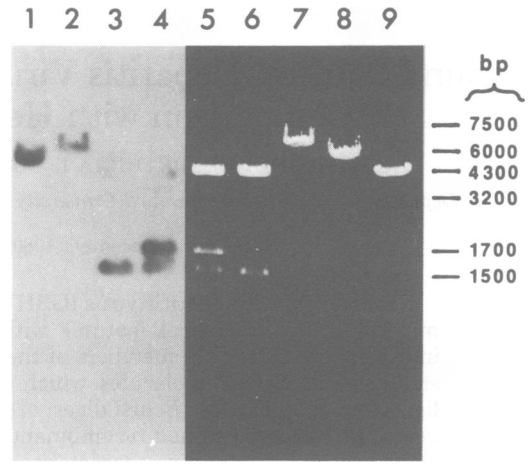

FIG. 1. *Restriction analysis of cloned GSHV DNA. Hybrid plasmids pGS2 and pGS11 were analyzed by electrophoresis in agarose gels after digestion with EcoRI and HindIII. DNA fragments were identified by ethidium bromide staining (5) (lanes 5 through 9) and then transferred to nitrocellulose filters (Millipore Corp.) by the method of Southern (16) for hybridization with GSHV [^{32}P]DNA radiolabeled in a virion DNA polymerase reaction (5, 15). Lanes 1 through 4 show the autoradiograph of the filter after hybridization. Lanes 1 and 8 contained HindIII-digested pGS2, lanes 2 and 7 contained HindIII-digested pGS11, lanes 3 and 6 contained EcoRI-digested pGS2, lanes 4 and 5 contained EcoRI-digested pGS11, and lane 9 contained HindIII-digested pBR322.*

(12, 13). The map of GSHV DNA and, for comparison, the map of DNA from HBV of HBV surface antigen subtype adw$_2$ are shown in Fig. 2. The 5′ end of the short strand was shown to be 1,950 bp in a clockwise direction from map point zero (one of the two *Eco*RI sites) in DNA oriented as in Fig. 2, and the 5′ end of the long strand was shown to be 2,250 bp in the same direction from map point zero. The distance of approximately 300 bp between the 5′ ends of the two strands of GSHV DNA is thus similar to that in HBV DNA (12). Although there are considerable differences in restriction sites in the two DNAs, the positions of the single-stranded region, the 5′ end of the short strand, and the nick in the long strand relative to one another are very similar in the two DNAs. Restriction maps of DNAs of HBV subtypes ayw$_3$ and adr (A. Siddiqui, J. J. Sninsky, S. N. Cohen, and W. S. Robinson, unpublished data) do not resemble the GSHV DNA restriction map more closely than does the map of HBV adw$_2$.

From the nucleotide sequence of HBV DNA (1, 8, 20) and the partial amino acid sequence of the major polypeptide of HBV surface antigen (9), the coding region for this polypeptide has

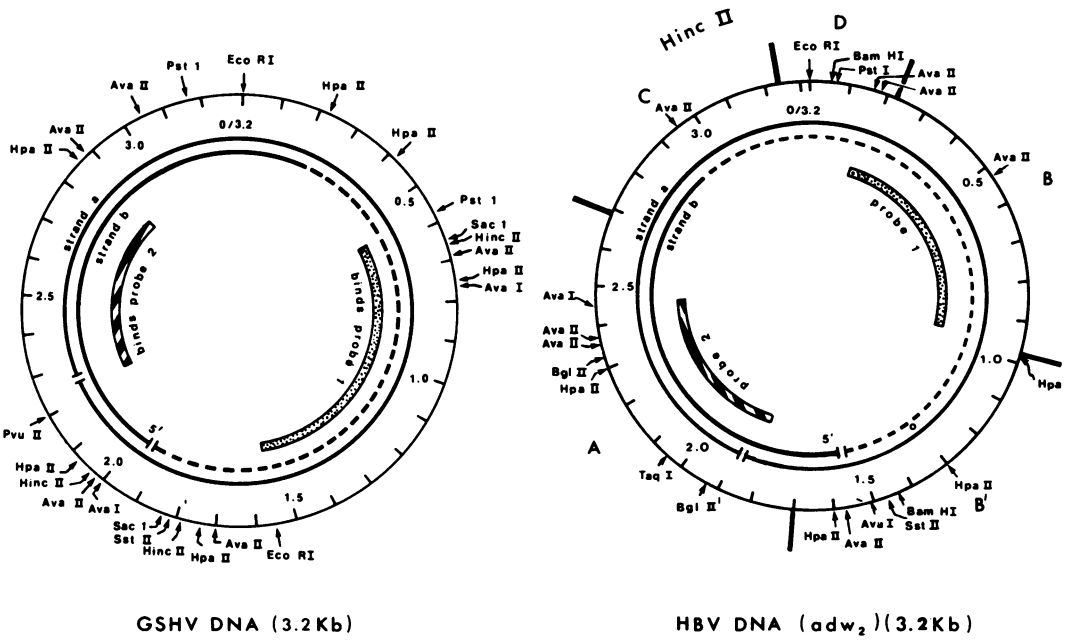

FIG. 2. *Physical structure and restriction endonuclease cleavage maps of GSHV and HBV DNAs. Restriction maps of GSHV DNA derived from pGSV11 and HBV DNA from clone pHBV1 (15) were determined by the method of Smith and Birnsteil (14). One of the EcoRI sites was chosen as the zero point for the GSHV DNA map. The inner circle represents the short (b) strand of the DNA, and the dotted line represents the region synthesized by the virion DNA polymerase. The region of the long strand (a) parallel to the dotted line represents the single-stranded region of virion DNA before repair by the virion DNA polymerase. Probe 1 consisted of the HBV DNA fragment between nucleotide pairs 166 (TacI site) and 967 (HpaI site) ^{32}P labeled by nick translation (10). Probe 2 consisted of the HBV DNA fragment between nucleotide pairs 1,807 (Hha I site) and 2,448 (AvaI site) ^{32}P labeled by nick translation. Probe 1 hybridized exclusively to the region of GSHV DNA between nucleotide pairs 574 (PstI site) and 1,550 (EcoRI site), and probe 2 hybridized exclusively to the region of GSHV DNA between nucleotide pairs 2,200 (PvuII site) and 3,870 (HpaII site).*

been located in the short strand between 161 and 839 nucleotides in a clockwise direction from the zero map point (the single *Eco*RI site) in HBV DNA (see Fig. 2). Another open reading frame in the same strand, between 1,820 and 2,480 nucleotides in the same direction from the *Eco*RI site, has been postulated to code for the major core polypeptide (19,000 daltons) of HBV because of its size (1, 8). Although no amino acid sequence is available for this polypeptide to identify its coding sequence in the DNA with certainty, a segment of the genome including the putative reading frame for this polypeptide has been translated in bacterial cells. The product was immunoprecipitable with anti-HBV core (8). To determine whether these nucleotide sequences in HBV DNA have homologous counterparts in GSHV DNA and, if so, to localize them in that DNA, restriction fragments of HBV DNA which contain either the coding sequence for the HBV surface antigen polypeptide (probe 1, Fig. 2) or that postulated for the core polypeptide (probe 2, Fig. 2) were radiolabeled by nick translation and used for hybridization with restriction fragments of GSHV DNA. Some DNA homology was anticipated for these probes, since the surface and core antigens of these viruses are each cross-reacting (2, 6) and the surface antigen polypeptides have common tryptic peptides (2).

Figure 3a shows the positions after gel electrophoresis of ethidium bromide-stained fragments generated by digestion of pGS11 DNA with *Pvu*II plus *Eco*RI (lane 1), *Pst*I plus *Eco*RI (lane 2), and *Eco*RI alone (lane 3), and Fig. 3b, lanes 1 through 3, respectively, show the blot hybridization (16) of HBV [^{32}P]DNA probe 1 with the same restriction fragments. Probe 1 was bound only by the 1,500-bp *Eco*RI B fragment (Fig. 3b, lane 3), which includes the single-stranded region of GSHV DNA as shown in Fig. 1, lane 3, and no binding of probe 1 to the *Eco*RI A fragment was observed. *Pst*I cleaves the *Eco*RI B fragment of GSHV DNA into 1,000- and 500-bp fragments (see Fig. 2), and probe 1 was specifically bound to the 1,000-bp fragment, and not

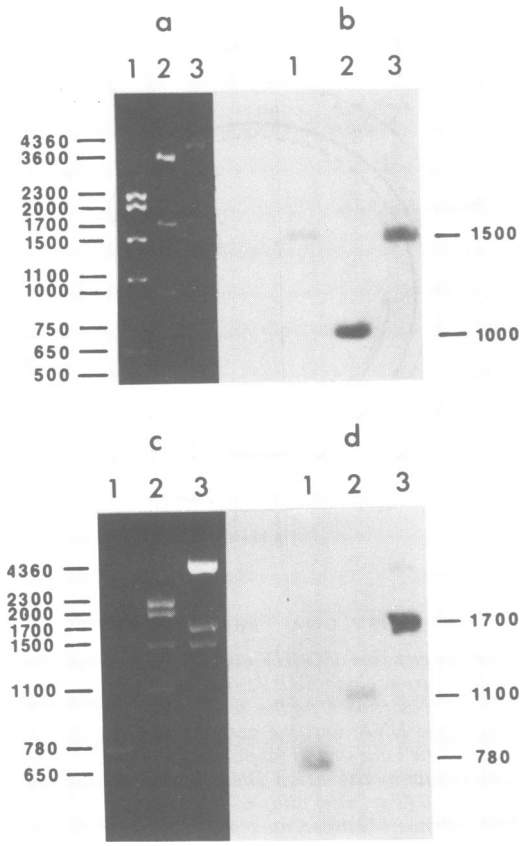

FIG. 3. *Hybridization of restriction fragments of GSHV DNA with HBV [^{32}P]DNA probes 1 and 2. (a, b) DNA fragments detected by ethidium bromide staining (5) after digestion of pGS11 with EcoRI and PvuII (lane 1), EcoRI and PstI (lane 2), and EcoRI alone (lane 3) and electrophoresis in a 1.4% agarose gel (a) and autoradiogram after transfer of the DNA from the same gel to a nitrocellulose filter by the method of Southern (16) and hybridization with probe 1 prepared as described in the legend to Fig. 2 (b). (c, d) Results of a similar experiment with the EcoRI A fragment of pGS11 digested with HpaII (lane 1), pGS11 digested with EcoRI and PvuII (lane 2), and pGS11 digested with EcoRI alone, with probe 2 used for the hybridization in (d).*

the 500-bp fragment, produced by double digestion with *Eco*RI and *Pst*I (Fig. 3b, lane 2). *Pvu*II cleaves the *Eco*RI A fragment (1,750 bp) into fragments of 1,100 and 650 bp. Probe 1 was bound only by the 1,500-bp *Eco*RI B fragment after double digestion of pGS11 DNA with these two enzymes (Fig. 3b, lane 1). It can be concluded from these experiments that HBV DNA probe 1 binds GSHV DNA only within the stippled region outlined in the physical map of this DNA in Fig. 2.

Fig. 3c shows ethidium bromide-stained fragments of the isolated *Eco*RI A fragment of the GSHV DNA insert in pGS11 after digestion with *Hpa*II (lane 1) and those of pGS11 DNA digested with *Eco*RI plus *Pvu*II (lane 2) or *Eco*RI alone (lane 3). Fig. 3d shows an autoradiogram of the same pGS11 fragments after blot hybridization with HBV [^{32}P]DNA probe 2. It is apparent that probe 2 was bound only by the *Eco*RI A fragment and not by *Eco*RI-B (Fig. 3d, lane 3). It was also bound by the 1,100-bp fragment generated by *Eco*RI-*Pvu*II digestion of pGS11 DNA. This fragment is part of the *Eco*RI A fragment (see Fig. 2). Finally, only the 780-bp fragment derived from *Hpa*II digestion of *Eco*RI-A appeared to contain nucleotide sequences recognized by probe 2. Its location within the GSHV DNA physical map is illustrated in Fig. 2. It can be concluded from these experiments that the HBV DNA probe 2 binds GSHV DNA only within the crosshatched region outlined in the physical map of GSHV DNA in Fig. 2.

Thus, the two specific regions of HBV DNA used here as probes, one of which appears to contain the coding sequence for the major HBV surface antigen polypeptide and the other of which possibly contains the core polypeptide coding sequence, have homology with GSHV DNA, and each of the homologous regions has approximately the same location relative to the single-stranded region, the 5′ end of the short strand, and the nick in the long strand in the two viral DNAs. These findings suggest that the two viruses have similar genetic organizations and that the unique physical features of the virion DNAs mentioned above may have the same locations relative to viral genes in these two viruses. The findings here strengthen other evidence cited above that these viruses are closely related and should be classified in the same virus group. It also appears that one of the uniquely located 5′ ends in the virion DNA may serve better as a reference point in constructing physical and genetic maps of these viral DNAs than restriction sites which appear to significantly vary in location in DNAs of different isolates of HBV (13) and among different viruses of this group as shown here.

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