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In Vitro Recombinants of Ground Squirrel and Woodchuck Hepatitis Viral DNAs Produce Infectious Virus in Squirrels

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Hepatitis B viruses of humans, woodchucks, ground squirrels, and ducks are similar biochemically but differ with respect to host range and pathogenicity. To pursue the genetic basis of these properties in the absence of a cell culture system for virus growth, we exploited the demonstrated infectivity of cloned hepatitis B virus DNA in whole animals. We constructed several recombinant molecules in vitro between cloned infectious genomes of woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) and assayed the recombinants for infectivity after intrahepatic injection in ground squirrels, which support growth of GSHV but not WHV. Two of the recombinants molecules initiated productive infection; in one recombinant genome, 76% of the coding region for the major surface glycoprotein of GSHV and for the overlapping portion of the presumptive gene for DNA polymerase was replaced by WHV DNA; in the other, 29% of the same coding domain was replaced by WHV DNA. These findings demonstrate the feasibility of generating viable recombinants of hepatitis B viruses from different animal species and suggest that the major host range determinants are not encoded within the surface antigen gene of these viruses.

Hepatitis B viruses of humans, woodchucks, ground squirrels, and ducks are structurally homogeneous, display strong hepatotropism, and replicate through an unusual mechanism that involves synthesis of small, relaxed circular DNA genomes from an RNA template (34, 35). Members of this virus family also exhibit a narrow host range and display different pathological potentials. For example, human hepatitis B virus, known to replicate only in humans and chimpanzees, and woodchuck hepatitis virus (WHV), confined thus far to Eastern woodchucks, are both associated with acute and chronic hepatitis. In contrast, ground squirrel hepatitis virus (GSHV), capable of growth in Beechey ground squirrels and certain chipmunks, appears to induce relatively minor pathological changes, even after long-standing infection (7, 13, 14, 36, 37). All three mammalian hepadnaviruses induce primary hepatocellular carcinoma in chronically infected individuals. In humans, primary hepatocellular carcinoma occurs infrequently and after a long incubation time (1); in contrast, most woodchucks chronically infected with WHV develop primary hepatocellular carcinoma as early as 17 months after infection (23, 34), whereas primary hepatocellular carcinoma formation in GSHV-infected squirrels occurs at a lower rate and after an incubation time of at least 4 years after infection (15; P.M., unpublished data).

Efforts to define and characterize the genetic basis of these intriguing biological properties of hepatitis B virus have been curtailed by the slow development of a cell culture system for virus propagation. Molecular cloning and nucleotide sequencing of the genomes of the four prototype viruses has unveiled the overlapping arrangement of 3 or 4 translatable domains, some of which have been assigned to known or postulated viral proteins, including the major surface glycoprotein (surface antigen [sAg]), the major polypeptide in the viral core (core antigen [cAg]), and a DNA polymerase activity believed to copy both DNA and RNA templates (5, 6, 11, 26, 28, 38). Cloned viral DNA has been useful for determining the mode of expression and protein products of the sAg gene and the preceding, contiguous open reading frame (the pre-S region) in cultured mammalian cells (3, 17, 29, 32), but unexplained constraints appear to operate upon the expression of other genes and replication of the viral genome in such cells.

We and others have recently shown that cloned genomes of hepatitis B viruses are infectious when introduced directly into the livers of appropriate host animals (27, 31, 40). As a result, the functional properties of open reading frames and other structural features of hepatitis B virus genomes can be investigated by exposing animals to genomes that have been altered through mutagenic or recombinatorial manipulations in vitro (28).

In this paper, we report the first demonstration that recombinants constructed in vitro between GSHV and WHV DNAs can direct synthesis of novel viruses. These viruses can replicate in ground squirrels despite the replacement of most of the GSHV sAg gene (and some of the overlapping polymerase gene) with the corresponding region of WHV DNA, implying that this region of the hepatitis B genome does not determine viral host range and is not sufficiently diverged between the two viruses to be incompatible with other components required for virus replication and packaging.

MATERIALS AND METHODS

Molecular clones. Clone pBA27-3 contains three head-to-tail genomes of GSHV cloned into the *Eco*RI site of a derivative of pBR322 (27). WHV clone pWHV81 was received from K. Denniston. pWHV81-2 is a derivative of pWHV81 containing two head-to-tail genomes cloned into the *Eco*RI site of pBR325.

DNA injections. DNA injections were performed as described previously (27), with the exception that each animal received equal amounts of DNA (10 μg) dissolved in phos-

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phate-buffered saline and DNA coprecipitated with calcium phosphate. For transfection of two woodchucks, the protocol for transfection of ground squirrels was applied, except that the amount of the inoculum was doubled.

Immunological assays. Serum samples obtained by cardiac puncture were monitored for ground squirrel hepatitis sAg and woodchuck hepatitis sAg by using the heterologous AUSRIA II kit (Abbott Laboratories, North Chicago, Ill.), originally designed for detection of sAg of human hepatitis B virus. The assay was performed as recommended by the manufacturer. Assays for detection of cAg antibodies were performed as described by Marion et al. (13).

Isolation and characterization of hepatic DNA. DNA was isolated from biopsied livers by phenol extraction with or without prior treatment with proteinase K (50 μg/ml). DNA samples (10 μg) were then subjected to electrophoresis in 1% agarose, transferred to nitrocellulose, and hybridized to a GSHV-specific ^{32}P-labeled probe (specific activity, 2×10^7 cpm/μg of DNA) (24, 30). Films were exposed for 12 to 36 h to Kodak XAR-5 film at −70°C.

Cloning of viral DNA from infected livers in lambda gt10. Hepatic DNA (40 μg) was digested to completion with *Eco*RI and subjected to electrophoresis in low-melting-point agarose (SeaPlaque; FMC Corp., Marine Colloids Div., Rockland, Maine). Under long-wave UV light, the gel was cut with a razor blade into eight segments containing DNA fragments ranging from 2.5 to 4.3 kilobases (kb). DNA was recovered with Elutip D (Schleicher & Schuell, Inc., Keene, N.H.) as described by the manufacturer. The presence of recovered GSHV DNA from the different gel segments was monitored by the dot blot hybridization method. DNA (120 ng) from a GSHV-positive fraction was ligated to 500 ng of *Eco*RI-digested lambda gt10 arms. The ligation products were packaged in vitro (12), and recombinant phage were plated on *Escherichia coli* NM514 on 15-cm (diameter) plates (19). Plaques were screened with a ^{32}P-labeled GSHV-specific probe. One positive clone was obtained from 30,000 plaques. After plaque purification, phage DNA was prepared by the plate lysate method (12), and the 3.3-kb insert was subsequently subcloned into a derivative of pBR322 (27) and into M13mp18 and M13mp19 for dideoxy nucleotide sequencing (2, 25).

RESULTS

Establishing the infectivity of cloned parental viral genomes. To construct in vitro recombinants between the GSHV and WHV genomes, we used a cloned species of GSHV DNA, strain 27, which has been fully sequenced and shown to be infectious in ground squirrels when injected intrahepatically (26, 27), and WHV clone WHV81 (9). To establish that the WHV DNA in clone pWHV81-2 is also infectious in its natural host, a mixture of recircularized *Eco*RI-cleaved DNA and uncut plasmid DNA containing the dimeric array was introduced directly into the livers of two male Eastern woodchucks during surgery, as described in Materials and Methods. sAg and virus-specific DNA were detected in serum samples 7 weeks later in one animal and 14 weeks later in the other, indicating that the cloned DNA had generated production of infectious virus. Thus, clone pWHV81-2 was shown to be a suitable substrate for the construction of recombinant genomes.

The host range difference between GSHV and WHV: WHV does not grow in ground squirrels. Hepadnaviruses typically manifest a relatively narrow host range, limiting their infectivity to a few phylogenetically related species. For example, human hepatitis B virus grows in humans and chimpanzees; GSHV grows in ground squirrels, certain chipmunks, and, as discovered recently and particularly relevant to this study, Eastern woodchucks (C. Seeger, D. Ganem, H. E. Varmus, and B. Tennant, unpublished data). Because we wished to use viral determinants of host range as one marker to assess the consequences of recombining the genomes of GSHV and WHV, it was important to establish whether parental WHV could grow in Beechey ground squirrels.

TABLE 1. Infectivity of the cloned genomes of GSHV and WHV in ground squirrels[a]

DNA and animal no.	sAg	Anti-cAg
GSHV		
372	−	+
374	ND	ND
383	+	+
385	−	ND
386	+	+
WHV		
366	−	ND
368	−	−
369	−	−
370	−	−
371	−	+
373	−	−
376	−	−
379	−	−
382	−	ND
387	−	−
388	−	−
389	−	−
390	−	ND
391	−	−

[a] Ground squirrels were injected with viral DNA as described in Materials and Methods. Clones pBA27-3 and pWHV81-2 served as sources of GSHV and WHV DNAs as described in the text. Serum samples (100 μl) obtained by cardiac puncture from each animal were analyzed with the AUSRIA II kit for the occurrence of sAg and for anti-cAg antibodies as previously described (13). Animals were monitored for sAg for more than 30 weeks and for anti-cAg for 11, 18, and 26 weeks after DNA infection. The three tests for anti-cAg gave the same results for all animals save 386, for which only tests 2 and 3 were positive. All animals were negative for sAg and anti-cAg before inoculation, with the exception of animal 371, which was positive for anti-cAg. ND, Not determined. Animal 374 died 13 weeks after DNA inoculation.

To examine this issue, we inoculated 14 sAg-negative ground squirrels with pWHV81-2 as described in Materials and Methods. Five additional animals received GSHV clone pBA27-3 and thus served as a positive control (Table 1). Two of the five animals inoculated with GSHV DNA exhibited surface antigenemia within 11 to 14 weeks, in accord with similar results obtained in our previous studies (27). Over an incubation period of 26 weeks, sAg was not observed in sera of any of the 14 squirrels inoculated with WHV DNA.

Since the appearance of anti-cAg antibodies is generally a more sensitive marker of hepadnavirus infection than is sAg (13), we examined whether any of the sAg-negative animals developed anti-cAg antibodies. Of the 11 sera assayed from animals inoculated with WHV DNA, 10 were negative for anti-cAg antibodies for 26 weeks (Table 1). The single positive animal had a detectable titer against cAg before DNA inoculation, indicating infection by GSHV before capture. In summary, ground squirrels appeared to be resistant to WHV infection under the described experimental conditions.

Construction of recombinants between infectious clones of GSHV and WHV DNAs. Our previous analysis of the nucle-

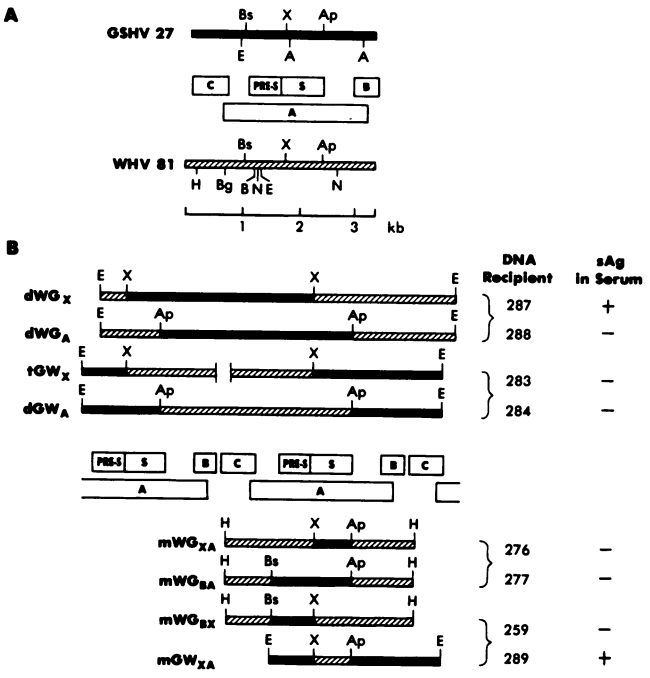

FIG. 1. Parental and recombinant hepatitis B virus DNAs. (A) Linearized forms of GSHV27-3 (27) and WHV81-2 DNAs are shown with the restriction sites used for the construction of recombinant molecules (above the bars) and for distinction between the two viruses (below the bars). The maps are arranged to begin on the left with the first AUG of the open reading frame for cAg (C), and the positions of that and other reading frames (for sAg and presurface [S and pre-S] and for proteins A [polymerase] and B) are indicated by open boxes between the genomes. (B) The indicated recombinant molecules were formed, as described in the text, by using the restriction endonuclease sites shown above each line, with the recombinants aligned with boxed open reading frames. The denoted combinations of recombinants were injected intrahepatically into ground squirrels, and serum samples were periodically monitored for the appearance of sAg with the heterologous AUSRIA II test (see Materials and Methods). The symbol + means that a P/N ratio of greater than 2 was observed in multiple tests. Abbreviations: Bs, *Bst*XI; X, *Xba*I; Ap, *Apa*I; E, *Eco*RI; A, *Ava*I; H, *Hind*III; B, *Bam*HI; N, *Nci*I.

otide sequence of GSHV DNA (26) demonstrated extensive homology with the published sequence of a clone of WHV DNA (5). To locate common restriction endonuclease cleavage sites in regions of the viral genomes suitable for generation of recombinant molecules, we subjected clone pWHV81-2 to extensive mapping of recognition sites likely to be shared with GSHV and present only once per genome; three such sites that proved to be useful in subsequent experiments—sites for *Bst*XI, *Xba*I, and *Apa*I—are shown in Fig. 1A. In addition, homology between the genomes in the regions of these sites was assured by partial nucleotide sequencing of pWHV81 (see Fig. 3 and 4; unpublished data) and confirmed by the published sequence of WHV81 (9).

Two strategies were used to construct recombinant genomes: one designed to produce a head-to-tail dimeric insertion in which one type of genome interrupts the other, and a second designed to yield a recircularized monomer composed of sequences from both parental genomes. We used both strategies because we were unable to determine in our earlier experiments whether oligomers or recircularized monomers were responsible for the infectivity of GSHV DNA; furthermore, if oligomers were infectious, it was uncertain whether they initiated infection by serving as a template for synthesis of viral RNAs or by undergoing homologous recombination to generate monomeric circles. Consequently, the recombinant dimers were formed so that viruses produced from primary transcripts of hybrid dimers or from recircularized monomers could be distinguished on the basis of the transcriptional program of GSHV (see Discussion).

To form the recombinant dimers, the plasmids carrying oligomers of GSHV and WHV DNAs were cleaved with either *Xba*I or *Apa*I, and the released 3.3-kb linear, monomeric fragments were isolated from agarose gels and ligated to larger fragments containing a plasmid vector joined to an interrupted heterologous genome. This approach produced the plasmids called dWGx, dWGa, tGWx, and dGWa, which contain the expected head-to-tail array of recombinant dimers; tGWx is unusual since two copies of the *Xba*I-linearized genome of WHV were apparently inserted during the ligation reaction (Fig. 1B). The plasmids bearing oligomeric recombinants were used for transfection studies without further manipulations.

To form the recircularized monomers, the dimeric recombinants described above were cleaved with an enzyme (either *Bst*XI or *Xba*I) different from that used to produce the dimeric molecules, recircularized by ligation at low concentration, recloned in pBR322 at either the *Hin*dIII or the *Eco*RI site for physical characterization, and then released from the vector and recircularized before transfection. For example, mWGxa was made by cleaving dGWa with *Xba*I, recircularizing the recombinant genome that contains a short region of GSHV DNA (from the *Xba*I to the *Apa*I site) joined to WHV DNA, and recloning after cleavage at the single *Hin*dIII site in WHV DNA (Fig. 1B). mWGba was similarly derived from dGWa, mWGbx was from dWGx, and mGWxa was from dWGa.

Infectivity of GSHV-WHV recombinant DNAs. Ground squirrels trapped in the vicinity of Palo Alto, Calif., and negative in heterologous tests of serum for hepatitis B sAg or antibody against sAg served as recipients for intrahepatic injections of the recombinant DNAs described in the preceding section. Since our supply of animals was limited and it was difficult to predict which recombinants, if any, were likely to be infectious, each animal received two kinds of recombinant genome, four of the animals receiving oligomeric recombinants and four receiving recircularized monomeric recombinants. The combinations used in each animal were chosen to reduce or prevent the possibility that intermolecular recombination during the transfection procedure would produce a wild-type, parental genome.

Treated animals were followed periodically for the appearance of surface antigenemia as a manifestation of successful infection. At 18 and 20 weeks after transfection, animals 287 (a recipient of two recombinant dimers) and 289 (a recipient of two recircularized monomeric recombinants) displayed sAg in their serum and were subjected to detailed study. The other animals remained negative for sAg for at least 40 weeks after transfection. To assure that the squirrels used for this study were susceptible to virus infection, five of the six negative animals were challenged with serum containing infectious GSHV (data not shown). Four of the five animals (283, 288, 276, and 277) developed transient surface antigenemia, indicating that they were susceptible hosts for DNA infection.

Recombinant GSHV-WHV viruses established characteristic infections in the livers of animals 287 and 289. sAg was

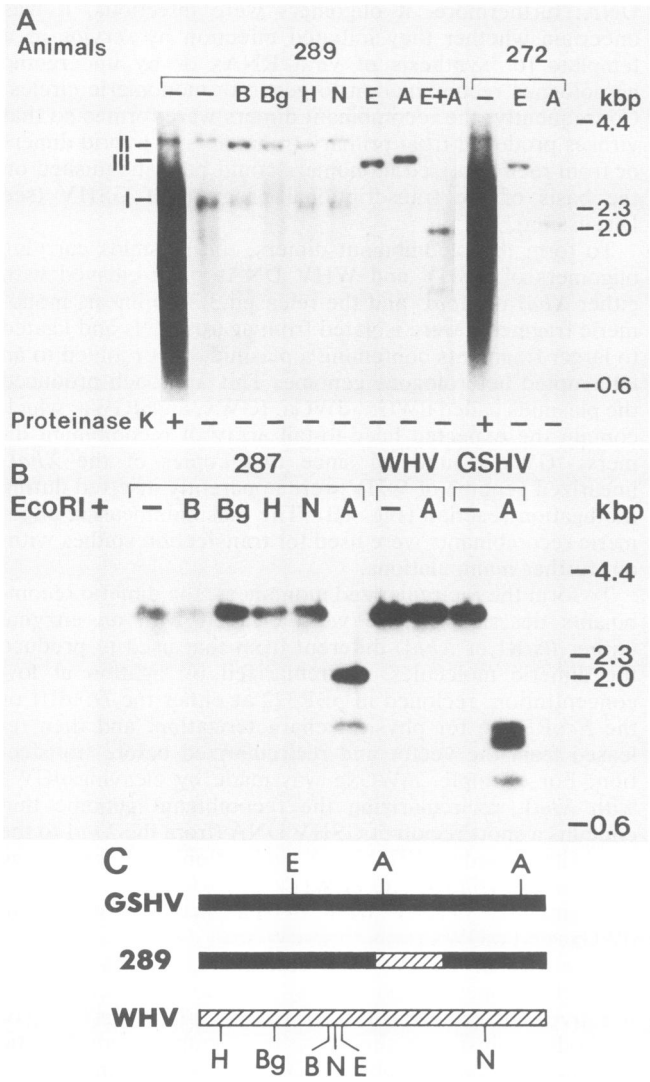

FIG. 2. Demonstration by restriction mapping that viral DNAs in livers of transfected animals are recombinants between WHV and GSHV DNAs. (A) Restriction site analysis of viral DNAs from animals 289 and 272. DNAs from liver biopsies of ground squirrels transfected with recombinants mWGbx and mGWxa (289) or wild-type GSHV DNA (272) were prepared either with (+) or without (−) proteinase K digestion before phenol extraction, electrophoresed through a 1% agarose gel undigested (−), or cleaved with restriction enzymes (B, *Bam*HI; Bg, *Bgl*II, H, *Hin*dIII; N, *Nci*I; E, *Eco*RI; A, *Ava*I). DNA was then transferred to nitrocellulose sheets and annealed to a GSHV-specific, nick-translated probe (specific activity, 2×10^7 cpm/μg of DNA). The positions expected for covalently closed circles (I), open circles (II), and linear duplex DNA of 3.3 kb (III) are indicated. The migration of forms II and III was affected by the extent to which liver DNA was cleaved by each restriction enzyme. kpb, Kilobase pairs. (B) Restriction site analysis of a cloned DNA isolate from the liver DNA of animal 287. Plasmid DNAs (100 ng) containing cloned viral DNA from the liver of the ground squirrel transfected with recombinants dWGx and dWGa (animal 287) and parental WHV and GSHV clones were cleaved with *Eco*RI to release viral DNAs from the vector, subsequently exposed to the indicated restriction enzymes (see above), electrophoresed in 1% agarose gels, and detected as for panel A. (C) Positions of restriction sites used to determine recombinant genomes. Sites in GSHV (solid bars) and WHV (striped bars) DNAs useful for interpretation of the experiments shown in panels A and B are shown, with a representation of the recombinant genome propagated in animal 289.

detectable in the serum of animal 287 for 10 weeks (until it died from unknown causes) and in the serum of animal 289 for 3 to 4 months, implying that prolonged virus infection, similar to that observed after infection of ground squirrels with GSHV, had occurred after transfection with recombinant genomes. We and others have previously shown that two major populations of intrahepatic, virus-specific DNA are present after infection and after transfection with wild-type hepatitis B virus DNA: (i) highly heterogeneous replicative intermediates, with a preponderance of minus (first)- over plus (second)-strand DNA, covalently joined to protein and (ii) closed and nicked circular duplexes unlinked to protein (16, 18, 39). To confirm that the expected replicative forms of hepatitis B virus DNA were abundant in liver cells, DNA was prepared from the liver of animal 289, with and without proteinase K treatment before phenol extraction. The abundant heterogeneous forms were readily observed in the sample deproteinized before phenol extraction (Fig. 2A, left-hand lane), as in the liver of animal 272, which was transfected with wild-type GSHV DNA (Fig. 2A, third lane from the right).

To detect the circular species and use them as substrates for analysis of restriction sites, samples were treated with phenol without prior exposure to protease (Fig. 2A). DNA migrating in the expected positions for forms I and II of ca. 3.3 kb were observed. In accord with the composition of only one of the two recircularized recombinant monomers injected into animal 289 (mGWxa), the circular forms from the liver of this animal were resistant to cleavage with several endonucleases that recognize only WHV DNA (*Bam*HI, *Bgl*II, and *Hin*dIII, and *Nci*I) but were cleaved to yield genome length linear DNA with an enzyme, *Ava*I, that recognizes two well-separated sites in GSHV DNA and no sites in WHV DNA. Double digestion with *Eco*RI and *Ava*I produced fragments of 2.0 and 1.3 kb, as expected if the sAg-coding region of GSHV DNA and its *Ava*I site were replaced by WHV DNA extending from the *Xba*I to the *Apa*I site (Fig. 2A and C). Thus, restriction site analysis of circular viral DNA in animal 289 was inconsistent with that of mWGbx and indistinguishable from that of mGWxa. mGWxa contained a GSHV genome with a woodchuck hepatitis virus sAg substitution between the *Xba*I and *Apa*I sites within the sAg-coding region and the overlapping region of the A gene (Fig. 1). An *Alu*I polymorphism distinguishing the GSHV and WHV DNAs 126 base pairs (bp) 5′ of the *Apa*I site was used to confirm the presence of a WHV sequence in the *Xba*I-*Apa*I region of the genome replicating in animal 289 (data not shown).

Examination of the liver of animal 289 5 months after infection showed no significant histopathological abnormalities (data not shown). The animal died from unknown causes after sAg levels had declined 6 months after infection.

In the case of animal 287, one of the dimers, dWGa or dWGx, served as a template for virus production. Though the restriction pattern of the DNA from animal 287 was indistinguishable from that of animal 289 (data not shown), suggesting a similar substitution of WHV DNA in a GSHV background, the exact junctions between GSHV and WHV sequences could not be determined. Therefore, we undertook molecular cloning of the putative recombinant viral genome and determination of part of its nucleotide sequence.

Analysis of GSHV-WHV recombinant DNA cloned from the liver DNA of animal 287. DNA extracted from the liver of animal 287 was cloned from a size-selected fraction of an *Eco*RI digest into lambda gt10 as described in Materials and Methods. The restriction pattern of the cloned isolate was,

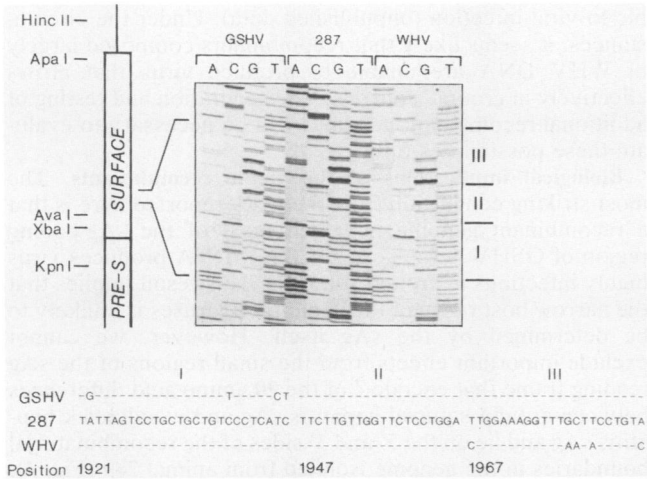

FIG. 3. Nucleotide sequence analysis of the junction between GSHV and WHV segments in the recombinant molecule from the liver of animal 287. The nucleotide sequence of cloned viral DNA from ground squirrel 287 was determined from the *Kpn*I site at position 1610 in the pre-S region to the *Hinc*II site at position 2506, 3′ of the sAg gene, by using the dideoxynucleotide method (24). The autoradiograph shows the sequencing ladder between positions 1920 and 1990 (within the sAg region), comparing the recombinant genome from animal 287 with GSHV clone 27 and WHV clone 81. The nucleotide sequence of the 287 clone presented below reveals that segment I (to position 1947) is identical to WHV DNA, that segment III (from position 1967) is identical to GSHV DNA, and that the recombinatory junction must reside within the 20 bp (region II) common to all three clones.

as expected, indistinguishable from that of viral DNA from animal 289 (Fig. 2B). The nucleotide sequence of the recombinant DNA from animal 287 was determined from the *Kpn*I site in the presurface region to the *Hinc*II site 391 bp 3′ of the translation stop codon in the sAg gene. The 5′ *Xba*I junction present in the dimer recombinant dWGx is preserved in the recombinant genome. The 3′ junction between the GSHV and WHV genomes is novel and must have been generated in vivo. It is located 186 to 206 bp downstream of the *Xba*I site at positions 1947 to 1966, within a 20-bp region of identity between the genomes of the two viruses (Fig. 3). A model for production of this recombinant is considered in the Discussion.

Nucleotide and amino acid substitutions in animals 287 and 289. Figure 4 displays the amino acid sequence differences between GSHV and WHV within the substituted segments in the recombinant viruses replicating in animals 287 and 289. A 9% divergence in nucleotide sequence exists between GSHV and WHV DNA within the *Xba*I-*Apa*I fragment, the segment exchanged in the virus replicating in animal 289. The resulting amino acid changes for the overlapping gene A and sAg coding regions are presented in Fig. 4. As a consequence of the *Xba*I-*Apa*I substitution in mGWxa, 13 (8%) of 170 amino acids were changed in surface frame and 21 (12%) of 170 amino acids were changed in the overlapping gene A frame. The shorter substitution in animal 287 resulted in a three-amino-acid difference in each open reading frame.

DISCUSSION

We have demonstrated the feasibility of making viable recombinants in vitro between the genomes of two mammalian hepatitis B viruses with different host ranges and pathogenic potentials. We have shown that at least two of eight engineered GSHV-WHV recombinant molecules initiated infections in ground squirrels typical of those that follow GSHV-initiated infection, that substantial nucleotide sequence exchanges between the two genomes are compatible with replication, expression, and packaging of the recombinant viral genomes, that most of the domain encoding the viral sAg can be exchanged without reversal of host range restrictions, and that homologous recombination within tandem dimers used for transfection can influence the boundaries of the exchanged regions in a viable recombinant genome. Ultimately, the approach we have taken may permit definition of the sequences responsible for differences in the biological behavior of these viruses.

Designing viable recombinants. Since it was uncertain at the outset whether any recombinant genomes would be infectious and since our supply of animals is limited, we manufactured recombinant monomers in which the boundaries of exchanged regions were fixed and recombinant oligomers from which new recombinants could be derived in vivo by intramolecular, homologous recombination (see Fig. 5). In addition, each animal received intrahepatic injections of two recombinant molecules, providing an opportunity for intermolecular recombination to produce additional combinations of GSHV and WHV components. However, we obtained no evidence for this phenomenon even when recombination could produce dimers of GSHV DNA (e.g., in animals 287 and 288 [Fig. 1B]). Qualitative analysis of liver DNA from both animals revealed a single population of GSHV-WHV recombinant molecules, indicating apparent clonal outgrowth of the infective genome.

The recombinant genome recovered from the animal (289) that received recircularized monomers displayed a restriction map consistent with the prediction that one of the designed recombinants (mGWxa; Fig. 1B) had replicated.

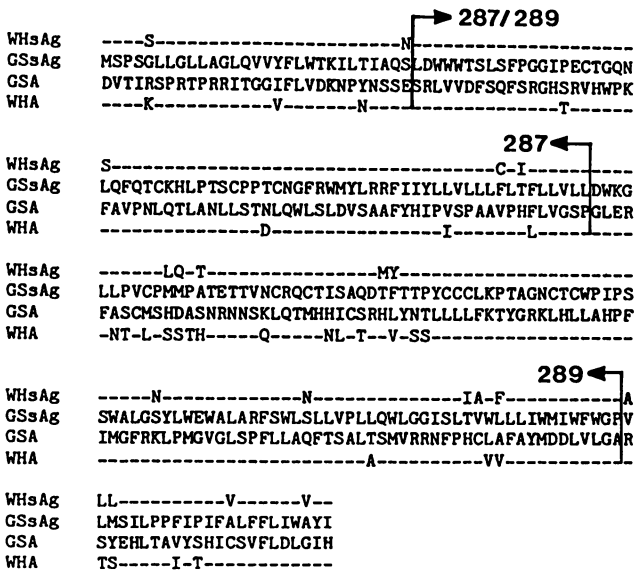

FIG. 4. Amino acid comparison of the sAg region and the overlapping gene A segment of GSHV 27 and WHV 81. The boundaries of the WHV fragments present in animals 287 and 289 are bracketed ([287]/[289]). Amino acids are indicated by the conventional one-letter code. GSA, GSHV A gene; WHA, WHV A gene; WHsAg, woodchuck hepatitis sAg; GSsAg, ground squirrel hepatitis sAg.

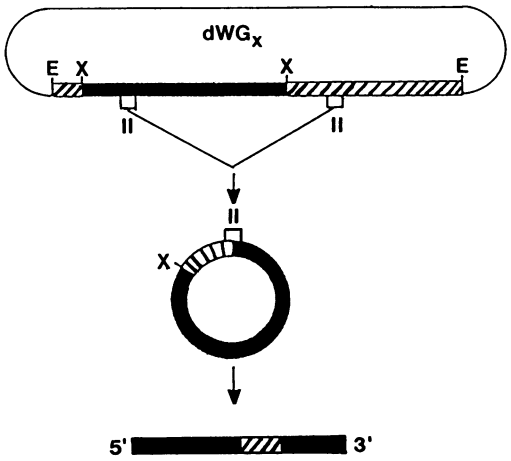

FIG. 5. Formation of the infective genome from the GSHV-WHV dimer dWGx. The genetic composition of dWGx is presented as shown in Fig. 1B. The sites for homologous recombination, as deduced from the sequence analysis in Fig. 3, are indicated (II). The resulting monomeric circle yields the RNA template for reverse transcription (pregenome) shown at the bottom of the figure (4, 28).

An infectious cycle was presumably initiated by transcription from circular DNA to produce an RNA template suitable for reverse transcription. However, since we did not subject the recombinant genome to nucleotide sequencing, we cannot rigorously exclude the possibility that small exchanges occurred before transcription between the two monomers introduced into the liver of animal 289.

Nucleotide sequencing was used, however, to determine the boundaries of the exchanged segments of the viral genome replicating in animal 287, since restriction mapping indicated that at least one of the boundaries was different from those in the injected recombinant dimers. At least two kinds of molecular events could produce novel recombinant genomes from the dimers: (i) a dimer could serve as a template for synthesis of hybrid RNA, which is then reverse transcribed, or (ii) homologous recombination within a dimer could produce a monomeric circle to serve as the template for RNA synthesis (Fig. 5). The 5′ end of GSHV pregenome RNA, the template for reverse transcription, has been mapped to the 5′ portion of the core open reading frame (4); inspection of the dimeric recombinants used to transfect animal 287 shows that a recombinant genome consistent with the species isolated from that animal cannot be generated by RNA synthesis from the dimeric templates. Instead, homologous recombination within dWGx most likely produced the infectious monomer as illustrated in Fig. 5.

The two viable recombinant genomes that we recovered both differed from parental genomes in the sAg-coding region and the overlapping portion of the A (or polymerase) reading frame. It would be premature, however, to conclude that other regions of the genome are more vulnerable to inactivation by recombination. The numbers of animals and recombinants tested was small, the efficiency with which acute infection is established even after virus infection is significantly less than 100% (generally 50 to 75% in our series), and viable recombinants that grow to lower titer than does wild-type virus might be undetectable in our assays or obscured by the growth of more effective recombinants. On the other hand, we showed by subsequent challenge with GSHV that at least four of the six animals that did not become infected with recombinant genomes were susceptible to viral infection (unpublished data). Under the circumstances, it seems likely that recombinants composed largely of WHV DNA are unable to produce virus that grows effectively in ground squirrels. The generation and testing of additional recombinant genomes will be necessary to evaluate these possibilities fully.

Biological implications of the viable recombinants. The most striking conclusion from the work reported here is that a recombinant genome in which most of the sAg-coding region of GSHV is replaced by WHV DNA produces virus highly infectious in ground squirrels. This result implies that the narrow host range of the hepatitis B viruses is unlikely to be determined by the sAg itself. However, we cannot exclude important effects from the small regions of the sAg reading frame that encode 7 of the 20 amino acid differences between ground squirrel hepatitis sAg and woodchuck hepatitis sAg and lie on the 5′ and 3′ sides of the recombinatorial boundaries in the genome isolated from animal 289 (Fig. 4).

The suggestion that host range determinants reside outside the sAg gene is in accord with hypotheses favoring a role for two proteins that are encoded by both the presurface and surface domains and found in virus particles (Dane particles) (8, 21). Synthesis of these proteins, pre-S1 and pre-S2, is initiated at AUGs positioned upstream of the initiation site for S protein and in the same reading frame (33; Fig. 1A). In the case of human hepatitis B virus, these proteins, but not surface (S) protein, include a region thought to mediate interaction with cellular receptors (20) and to be responsible for binding to in vitro polymerized albumin in a species-specific manner (10, 22). Either of these properties might be related to the host range capabilities of hepadnaviruses. Production of additional recombinant genomes in which portions of the presurface domain are exchanged and challenge of larger cohorts of animals with such recombinants will be required to examine these hypotheses more rigorously.

Finally, the viability of GSHV-WHV recombinants might be exploited to map determinants of viral pathogenicity. This approach is likely to be useful if chronic infection of woodchucks with GSHV elicits significantly less inflammation or fewer tumors compared with WHV infection, thereby implying that viral rather than host factors are the primary determinants of pathogenicity. A test of this issue is currently in progress. Similarly, it should be possible to follow the pathological consequences of chimeric genomes in ground squirrels to see whether replacement of GSHV sequences with regions of WHV DNA augments the oncogenic or inflammatory properties of GSHV.

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