An Infectious Clone of Woolly Monkey Hepatitis B Virus

Robert E. Lanford,1,2,* Deborah Chavez,1 Azeneth Barrera,1,2 and Kathleen M. Brasky3

Department of Virology and Immunology1 and Department of Comparative Medicine,3 Southwest National Primate Research Center, Southwest Foundation for Biomedical Research, San Antonio, Texas 78227, and Department of Microbiology, University of Texas Health Science Center, San Antonio, Texas 782292

Received 30 January 2003/Accepted 30 April 2003

Members of the Hepadnaviridae family have been isolated from birds, rodents, and primates. A new hepadnavirus isolated from the woolly monkey, a New World primate, is phylogenetically distinct from other primate isolates. An animal model has been established for woolly monkey hepatitis B virus (WMHBV) by using spider monkeys, since woolly monkeys are endangered. In this study, a greater-than-genome length construct was prepared without amplification by using covalently closed circular DNA extracted from the liver of an infected woolly monkey. Transfection of the human liver cell line Huh7 with WMHBV DNA resulted in the production of viral transcripts, DNA replicative intermediates, and secreted virions at levels similar to those obtained with an infectious human HBV clone, demonstrating that the host range restriction of WMHBV is not at the level of genome replication. WMHBV particles from the medium of transfected cultures initiated an infection in a spider monkey similar to that obtained with virions derived from woolly monkey serum. In an attempt to adapt the virus for higher levels of replication in spider monkeys, immunosuppressed and newborn animals were inoculated. Neither procedure produced persistent infections, and the level of viral replication remained several logs lower than that observed in persistently infected woolly monkeys. These data demonstrate the production of an infectious clone for WMHBV and extend the characterization of the spider monkey animal model.

Hepatitis B virus (HBV) infections represent a worldwide health problem. Although acute HBV infection in adults is normally self-limiting, serious liver disease, including fulminating hepatitis, often develops. In addition, approximately 5 to 10% of the individuals infected as adults and 95% of those infected at birth become chronically infected. Persistently infected individuals are at risk for cirrhosis, end stage liver disease, and hepatocellular carcinoma (2). Most of our understanding of the pathogenesis and replication of HBV has been obtained by the use of related hepadviruses and their animal models, including duck HBV (12), woodchuck hepatitis virus (17), ground squirrel hepatitis virus (11), arctic ground squirrel hepatitis virus (20), and stork hepatitis virus (14). Infection of chimpanzees with human HBV is the only primate model for the analysis of HBV infections. Recently, a number of hepadnaviruses have been isolated from nonhuman primates. Most of the nonhuman primate isolates were from apes and clustered phylogenetically with human HBV isolates. Viruses have been isolated from gibbons (8, 13), chimpanzees (4, 5, 10, 19, 21), and orangutans (22). The origins of these viruses remain controversial. Although cross-species transmission from humans cannot be ruled out, chimpanzees from different geographical regions appear to be infected with distinct isolates (4), lending support to the concept that these viruses may have evolved with their hosts. However, the hepadnavirus isolated from woolly monkeys, woolly monkey HBV (WMHBV), clearly represents a new member of the Hepadnaviridae family (7). The ability to infect spider monkeys with this virus provided a new nonhuman primate model for the analysis of hepadnavirus infections and pathogenicity.

The WMHBV genome has the same genetic organization as human HBV. The core open reading frame (ORF) is the most conserved region of the genome, with approximately 15 and 24% divergence at the amino acid and nucleotide levels, respectively, in comparison to the human HBV core. The X region exhibits approximately 35% divergence between WMHBV and human HBV at the amino acid level, and the region of overlap between the pre-S1 and polymerase spacer regions also exhibits a high degree of divergence (30 to 32%). Nonetheless, recent studies have suggested that the WMHBV core and polymerase functions can substitute for the human HBV functions in genome replication (9) and that the WMHBV X region possesses transcriptional transactivating properties similar to those of the human HBV X protein (15).

In this report, we describe the construction of an infectious clone of WMHBV. The clone was derived by direct cloning from liver DNA of an infected woolly monkey without PCR amplification. Following transfection, the clone initiated replication in the human liver cell line Huh7 at levels similar to those of human HBV, and the virus secreted into the culture medium was infectious in spider monkeys. Attempts to improve the animal model by adaptation of the virus for better replication in spider monkeys, by either immunosuppression or inoculation of newborn animals, was not successful despite the induction of viremia of greater duration.

MATERIALS AND METHODS

Animals. Black-handed spider monkeys (Ateles geoffroyi) were housed at the Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research. Animals were cared for by members of the Department of Comparative Medicine in accordance with the Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Institutional Animal Care and Use Committee. Black-handed spider monkeys (Ateles geoffroyi) were housed at the Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research. Animals were cared for by members of the Department of Comparative Medicine in accordance with the Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Institutional Animal Care and Use Committee.

* Corresponding author. Mailing address: Department of Virology and Immunology, Southwest National Primate Research Center, Southwest Foundation for Biomedical Research, 7620 N.W. Loop 410, San Antonio, TX 78227. Phone: (210) 258-9442. Fax: (210) 670-3229. E-mail: rlanford@icarus.sfbfr.org.
Care and Use Committee. FSK06 (tacrolimus or Prograf) was purchased from Fujisawa Pharmaceutical Company. The animals were given FSK06 orally twice daily at 100 μg/kg. The trough levels in blood were measured several times to ensure that therapeutic levels were being obtained. The enzyme-linked immuno- sorbent assay (ELISA) of p2 was performed to determine the anti-plasmid backgrounds, pBluescript SK HBV GGL clone has been previously described (1, 3, 18) and was derived from the WM GGL construct, spanning nucleotide positions 1575 to 1980. The human Not cloning of an the clone was produced without the use of PCR amplification by digestion with HindIII and ApaI (nucleotide position 1575–1980) fragment into a plasmid backbone (pBluescript, and pZero). The nucleotide numbering of the HBV genome corresponds to the HBV sequence convention used for both WMHBV and HBV. The sequence of the GGL clone was compared with that of the full-length human HBV and WMHBV DNAs. For Hu-SG pZ and Hu-SG pBS, a WI fragment spanning nucleotide positions 2425 to 1987 was inserted into a plasmid backbone (pBluescript, and pZero). The nucleotide numbering of the HBV genome corresponds to the HBV sequence convention used for both WMHBV and HBV.

For analysis by the endogenous polymerase assay, core particles were immunoprecipitated as described above. The beads were washed twice with endogenous polymerase (EP) buffer (50 mM Tris-HCl [pH 7.4], 10 mM MgCl₂, 100 μM aminoguanidine, and 10 μg/ml pepstatin per ml and 1 ml M EDTA). The medium was supplemented with 1% Igepal to remove the envelope prior to immunoprecipitation. Core particles were immunoprecipitated for 16 h at 4°C with rabbit anti-core antibody bound to protein A agarose beads. Following immunoprecipitation, the beads were washed twice with EB buffer and treated with 1.5 U of micrococcal nuclease (USB, Cleveland, Ohio) for 30 min at 37°C.

Cells and transfections. The human hepatoma cell line Huh7 was maintained in Dulbecco modified Eagle medium (DMEM)-F12 medium (1:1) containing 10% fetal bovine serum, 2 mM glutamine, and 50 μg of gentamicin per ml. Huh7 cells were amplified with 500 μg/l of WM-SG pBS. For Hu-SG pZ and Hu-SG pBS, a BglII-to-BglIII fragment spanning nucleotide positions 2425 to 1870 was inserted into a BamHI-digested vector.

Cells and transfections. The human hepatoma cell line Huh7 was maintained in Dulbecco modified Eagle medium (DMEM)-F12 medium (1:1) containing 10% fetal bovine serum, 2 mM glutamine, and 50 μg of gentamicin per ml. Huh7 cells were amplified with 500 μg/l of WM-SG pBS. For Hu-SG pZ and Hu-SG pBS, a BglII-to-BglIII fragment spanning nucleotide positions 2425 to 1870 was inserted into a BamHI-digested vector.

Cells and transfections. The human hepatoma cell line Huh7 was maintained in Dulbecco modified Eagle medium (DMEM)-F12 medium (1:1) containing 10% fetal bovine serum, 2 mM glutamine, and 50 μg of gentamicin per ml. Huh7 cells were amplified with 500 μg/l of WM-SG pBS. For Hu-SG pZ and Hu-SG pBS, a BglII-to-BglIII fragment spanning nucleotide positions 2425 to 1870 was inserted into a BamHI-digested vector.

Cells and transfections. The human hepatoma cell line Huh7 was maintained in Dulbecco modified Eagle medium (DMEM)-F12 medium (1:1) containing 10% fetal bovine serum, 2 mM glutamine, and 50 μg of gentamicin per ml. Huh7 cells were amplified with 500 μg/l of WM-SG pBS. For Hu-SG pZ and Hu-SG pBS, a BglII-to-BglIII fragment spanning nucleotide positions 2425 to 1870 was inserted into a BamHI-digested vector.

Cells and transfections. The human hepatoma cell line Huh7 was maintained in Dulbecco modified Eagle medium (DMEM)-F12 medium (1:1) containing 10% fetal bovine serum, 2 mM glutamine, and 50 μg of gentamicin per ml. Huh7 cells were amplified with 500 μg/l of WM-SG pBS. For Hu-SG pZ and Hu-SG pBS, a BglII-to-BglIII fragment spanning nucleotide positions 2425 to 1870 was inserted into a BamHI-digested vector.

Cells and transfections. The human hepatoma cell line Huh7 was maintained in Dulbecco modified Eagle medium (DMEM)-F12 medium (1:1) containing 10% fetal bovine serum, 2 mM glutamine, and 50 μg of gentamicin per ml. Huh7 cells were amplified with 500 μg/l of WM-SG pBS. For Hu-SG pZ and Hu-SG pBS, a BglII-to-BglIII fragment spanning nucleotide positions 2425 to 1870 was inserted into a BamHI-digested vector.
RESULTS AND DISCUSSION

Construction of a WM GGL clone. The initial WMHBV sequence was amplified from viral DNA purified from the serum of an infected woolly monkey (7). This sequence was not assembled into a full-length clone, since it was found to contain a number of nucleotide changes in comparison to a clone derived without PCR amplification. The original sequence will be referred to as WMHBV-1. To avoid potential errors introduced by PCR, we decided to derive a clone directly from cccDNA extracted from the liver of an infected woolly monkey that died of fulminant hepatitis. Full-length WMHBV DNA was cloned by digestion at the unique HindIII site, and then a GGL clone was assembled as described in Materials and Methods. This clone was designated WMHBV-2 and differed from WMHBV-1 at 16 nucleotide and 15 amino acid positions.

Analysis of transcription of WMHBV-2 in Huh7 cells. Since no woolly monkey or spider monkey liver cell lines were available, the replication potential of WMHBV-2 was analyzed with the human liver cell line Huh7 and directly compared to that of an HBV infectious clone of the ayw serotype (18). Initially, the transcriptional patterns of the two viruses were compared. Huh7 cells were transfected with WMHBV-2 GGL (WM GGL) or HBV ayw GGL (Hu GGL), and cultures were harvested at various times over a 9-day period. A typical transcript pattern was observed by Northern hybridization for both viruses including prominent bands representing single-stranded (SS) and relaxed-circular (RC) DNAs. Identical patterns for replicative intermediates were observed with both viruses including prominent bands representing single-stranded (SS) and relaxed-circular (RC) DNAs. Identical patterns were observed, independently of the GGL vector used (pBS or pZero), but minor differences in the abundance of RC DNA between WMHBV and HBV were observed (Fig. 3).

To further examine the in vitro replication competence of which are poorly resolved on most gels. However, the level of the 2.4-kb transcript appeared to be reduced in the WM GGL-transfected cells. Analysis of a separate transfection at day 6 clearly indicated that the 2.4-kb transcript was reduced or absent in the WM GGL-transfected cells (Fig. 1, far right lanes).

We further examined this difference in transcript patterns by preparing GGL constructs of both WMHBV and HBV in the vectors pBluescript SK+ (GGL pBS) and pZero (GGL pZ) and by preparing subgenomic clones that encode the envelope proteins with their promoters and the poly(A) site (SG pBS and SG pZ). Transfection of these constructs into Huh7 cells and analysis by Northern hybridization confirmed that the 2.4-kb transcript for the WMHBV large envelope protein was not as apparent as the equivalent transcript from HBV (Fig. 2). Whether this difference was due to reduced transcription from the WM pre-S promoter in Huh7 cells or comigration of the transcript with the 2.1-kb transcript was not pursued, since it was determined that the WMHBV-2 construct was infectious in spider monkeys (see below).

Analysis of replication of WMHBV-2 in Huh7 cells. To examine the replication competence of the WMHBV-2 construct, Huh7 cells were transfected with WM GGL and Hu GGL and DNA replicative intermediates were examined in secreted virions. Medium was harvested 6 days posttransfection and treated with detergent to remove the envelope from particles. Core particles were immunoprecipitated with rabbit anti-core antibodies, and DNA extracted from the core particles was analyzed by Southern blot hybridization. Typical patterns for replicative intermediates were observed with both viruses including prominent bands representing single-stranded (SS) and relaxed-circular (RC) DNAs. Identical patterns were observed, independently of the GGL vector used (pBS or pZero), but minor differences in the abundance of RC DNA between WMHBV and HBV were observed (Fig. 3).
WMHBV-2 in Huh7 cells, endogenous polymerase assays were conducted in which the encapsidated reverse transcriptase extends the partially completed positive-strand DNA, allowing the incorporation of radiolabeled nucleotides. Huh7 cells were transfected with the GGL constructs, core particles were immunoprecipitated from detergent-treated medium with anti-core antibodies. Viral DNA was purified from the core particles and analyzed by Southern blot hybridization with a mixed probe containing both HBV and WMHBV sequences. The migration of single-stranded (SS) and RC DNAs is indicated. The values on the left are the molecular sizes, in kilobases, of the markers in lane M.

**VOL. 77, 2003 WMHBV INFECTIOUS CLONE 7817**

**FIG. 3.** Replication of WMHBV in Huh7 cells. Huh7 cells were transfected with the WMHBV-2 (WM) and HBV (Hu) GGL constructs, and medium was harvested 6 days posttransfection. Core particles were immunoprecipitated from detergent-treated medium with anti-core antibodies. Viral DNA was purified from the core particles and analyzed by Southern blot hybridization with a mixed probe containing both HBV and WMHBV sequences. The migration of single-stranded (SS) and RC DNAs is indicated. The values on the left are the molecular sizes, in kilobases, of the markers in lane M.

**FIG. 4.** Endogenous polymerase reaction of WMHBV particles. Huh7 cells were transfected with the WMHBV-2 (WM) and HBV (Hu) GGL constructs, and medium was harvested on day 3 (d3) and d6, and cells were harvested on d6. Core particles were immunoprecipitated from the cell lysates and detergent-treated medium with anti-core antibodies, and endogenous polymerase reactions were conducted with the core particles still bound to the immunobeads. Viral DNA was purified from the core particles and analyzed by gel electrophoresis and autoradiography.

**FIG. 5.** Infection of a spider monkey with WMHBV-2-derived particles. WMHBV particles were produced by transfection of Huh7 cells with WMHBV-2 GGL, and particles secreted into the medium were concentrated by ultrafiltration and ultracentrifugation. A spider monkey (14495) was inoculated intravenously with 10^12 particles and monitored by TaqMan PCR for the level of viremia (bar graph) and by ELISA for the levels of HBsAg (plus and minus signs at the top of the graph) and anti-HBcAg (line graph). OD, optical density.

Infectivity of WMHBV-2 in spider monkeys. To confirm the infectivity of the WMHBV-2 clone, virus produced in Huh7 cells following transfection with WM GGL was inoculated into a spider monkey (Fig. 5). A large-scale transfection was performed with ten 100-mm dishes, and the medium was concentrated by ultrafiltration. A membrane with a 300,000 molecular weight cutoff was used to eliminate most of the serum and cellular proteins present in the medium. Spider monkey 14495 was inoculated intravenously with approximately 10^12 genome equivalents (ge) of WMHBV as estimated by real-time, quantitative TaqMan PCR. Within 1 week postinoculation, viremia peaked at 2.2 × 10^5 ge/ml of serum. Viremia remained elevated until week 8, at which time viral clearance occurred. The HBsAg ELISA values exhibited a profile similar to that of viremia with high reactivity until week 6 and then negative values thereafter. Since baboons, rhesus monkeys, and tamarins inoculated with WMHBV were PCR negative by week 1 (data not shown), it can be concluded that the 6 weeks of viremia observed in spider monkey 14495 represents viral replication. In addition, seroconversion for antibodies to HBcAg and WMHBsAg occurred at week 8, at the time of viral clearance.
ance, which is another indicator of active replication. No significant rise in alanine transaminase occurred, indicating the absence of overt liver disease (data not shown). These data demonstrate that the WMHBV-2 clone is infectious in spider monkeys and induces an infection profile similar to that observed with woolly monkey serum containing WMHBV (7).

Effects of immunosuppression and inoculation at birth on the duration and level of WMHBV viremia in spider monkeys. The permissiveness of spider monkeys for infection with WMHBV has provided an animal model for this new hepadnavirus. We have not observed significant replication in other New World (tamarins) or Old World (baboons, rhesus macaques, and chimpanzees) primates. The spider monkey is a close relative of the woolly monkey (Fig. 6); thus, the data suggest that, similar to those of other hepadnaviruses, the host range of WMHBV is very narrow. The level of viremia in chronically infected woolly monkeys is very high. Quantification by TaqMan assay revealed that the mean viral load was $1.8 \times 10^7$ ge/ml for the seven carriers for which serum was available. No acute resolving infections of woolly monkeys have been monitored. This level of viremia is 3 to 4 logs higher than the peak viremia in spider monkeys inoculated with virus from the infectious clone ($2.2 \times 10^6$ ge/ml) or with woolly monkey serum ($1.1 \times 10^5$ to $1.3 \times 10^6$ ge/ml) (7). We reasoned that a virus more fit for replication in spider monkeys would emerge because of random mutations if viremia could be prolonged or if chronic infection could be induced. We also reasoned that if viral levels were being suppressed by the immune system, immunosuppression might increase the level of viremia and enhance the potential for adaptation. Therefore, in an attempt to increase the duration and level of viremia, a spider monkey was immunosuppressed during infection with WMHBV. Animal 14494 was maintained on FK506 beginning 3 weeks prior to inoculation and for 22 weeks postinoculation, when viral clearance was documented by two consecutive blood samples negative for WMHBV DNA and HBsAg reactivity. Viremia was detectable until week 18, with peak viremia at $5.1 \times 10^4$ ge/ml (Fig. 7). HBsAg reactivity was present in the serum until week 14, and seroconversion for both anti-HBcAg and anti-WMHBsAg antibodies occurred between weeks 22 and 35. Unfortunately, serum samples were not available at times between weeks 22 and 35. Although chronic infection was not attained in this animal, the duration of viremia was greater than that previously observed in immunocompetent animals (7). The number of animals that could be devoted to this approach was limited. Spider monkeys are not being bred for biomedical research at any primate center in the United States. For our studies, we obtained the few remaining animals from a colony previously housed at another facility. We are breeding additional animals for these studies, and several of these were used in the studies involving inoculation of newborn spider monkeys (see below). Nonetheless, in a single animal, immunosuppression yielded 18 weeks of viremia, in comparison to the 4 to 6 weeks of viremia observed in all of the previously inoculated immunocompetent spider monkeys. Although the extended duration of viremia may have been due to the genetic background of this animal, it was most likely a result of the immunosuppression. Rather than pursue this approach with additional animals, alternative strategies for the induction of chronicity were chosen.

One alternative strategy by which to achieve persistent infection is inoculation of animals at birth, since most humans exposed at birth develop chronic infections while adults normally clear the virus. Two spider monkeys (14333 and 16275) were inoculated at birth. Blood samples were infrequently ob-

![FIG. 6. Classification of primates. The classification scheme for primates is illustrated. The primates are divided into New World primates, Old World primates, and hominoids, which include the lesser and greater apes. The common names of many of the primate genera are shown. Note the close relationship between spider and woolly monkeys. Hepadnaviruses have been isolated from woolly monkeys, gibbons, orangutans, and chimpanzees.](http://jvi.asm.org/)
tained from these animals, since they were being carried by their mothers. The serum of the first animal (14333) was positive for viral DNA at months 2, 5, 9, and 10, but viral clearance occurred by month 14. Peak viremia was 2.9 × 10^4 ge/ml. The second animal (16275) was PCR positive at month 2 but negative by month 5. Thus, in neither case did inoculation at birth result in chronic infection or higher levels of viremia. However, since the duration of viremia was greater with both immune-suppression and inoculation at birth, additional efforts are warranted to improve the animal model for WMHBV.

The factors limiting the level of WMHBV viremia in spider monkeys are not apparent. Presumably, proper interaction with an essential cellular protein is lacking or reduced in spider monkeys. One possible explanation for the lack of adaptation to a more “fit” virus is that an overlapping essential function prevents the emergence of a virus with higher replication competence. The overlapping nature of the polymerase ORF with other ORFs and cis functions certainly constrains the evolution of hepadnaviruses. Surprisingly, the WMHBV infectious clone replicates in the human cell line Huh7 at levels similar to those of HBV. Although receptor interactions cannot be examined in Huh7 cells, it is unlikely that receptor interactions account for the reduced viremia. The lack of interaction between the receptor and WMHBV in spider monkeys would presumably completely block infection, while a reduced affinity for the virus would presumably still result in spread of the infection to all susceptible hepatocytes. Another possible factor that limits viremia is greater suppression of WMHBV replication by the innate immune system of spider monkeys. Further evaluation of this virus-host interaction may provide additional information about the factors that regulate hepadnavirus infections.

ACKNOWLEDGMENTS

This work was supported by grants RO1 AI46609 and P51 RR13986 from the National Institutes of Health.

REFERENCES