

## Hepadnavirus Infection in Captive Gibbons

ROBERT E. LANFORD,<sup>1\*</sup> DEBORAH CHAVEZ,<sup>1</sup> REBECA RICO-HESSE,<sup>1</sup> AND ALAN MOOTNICK<sup>2</sup>

*Department of Virology and Immunology, Southwest Regional Primate Research Center, Southwest Foundation for Biomedical Research, San Antonio, Texas 78227,<sup>1</sup> and International Center for Gibbon Studies, Santa Clarita, California 91380<sup>2</sup>*

Received 26 August 1999/Accepted 16 December 1999

**The recent isolation of a nonhuman primate hepadnavirus from woolly monkeys prompted an examination of other primates for potentially new hepadnaviruses. A serological analysis of 30 captive gibbons revealed that 47% were positive for at least one marker of ongoing or previous infection with a hepatitis B virus (HBV). The amino acid sequences of the core and surface genes of human and gibbon virus isolates were very similar. Phylogenetic analysis indicated that the gibbon isolates lie within the human HBV family, indicating that these HBV isolates most likely stem from infection of gibbons from a human source.**

Hepadnaviruses are classified into two genera representing isolates from birds (*Avihepadnavirus*) and rodents and humans (*Orthohepadnavirus*). The avihepadnaviruses include isolates from ducks (8) and heron (11), while rodent viruses have been isolated from the woodchuck (12), ground squirrel (7), and arctic ground squirrel (13). Until recently, the only primate hepadnavirus described was human hepatitis B virus (HBV). Recently, we isolated a hepadnavirus from a woolly monkey experiencing fulminant hepatitis at the Louisville Zoo (WMHBV) (5). A survey of captive woolly monkeys indicated that approximately 50% of the animals at different zoos were positive for at least one marker of current or previous infection with WMHBV. WMHBV is phylogenetically distinct from the human HBV family and thus represents the first nonhuman primate hepadnavirus. The isolation of WMHBV rekindled an interest in the possibility that other primate hepadnaviruses could be identified. Previously, the isolation of a hepadnavirus from a single chimpanzee (14) and a gibbon (10) had been reported. Phylogenetic analysis of the nucleotide sequences of these viruses suggested that they were members of the human HBV family, since they were more closely related to human genotypes A to E than were the human genotype F isolates (5). However, the isolation of a distinctive, nonhuman primate hepadnavirus (WMHBV) suggested that a more in-depth analysis of the gibbon viruses was warranted.

The genus *Hylobates* (gibbons) contains 11 species and 4 subgenera that occupy forests in distinct geographical regions in Southeast Asia, including eastern India, southern China, Vietnam, Cambodia, Burma, Thailand, Malaysia, Borneo, Java, and Sumatra. Examination of gibbons in the wild was not practical, so we chose to examine captive gibbons housed at the International Center for Gibbon Studies (Santa Clarita, Calif.). A total of 30 animals were examined, which represented six different species and three subgenera of gibbons (Table 1). The gibbons are housed individually or in small monogamous families, thus facilitating the evaluation of common exposures. None of the animals had been involved in any experimental procedures, and some were wild-caught animals. Serum from the animals was examined for the presence of HBV DNA by

PCR using primers to the core region that are conserved among all human HBV genotypes (5). Enzyme-linked immunosorbent assays (ELISAs) for HBsAg, anti-HBsAg, and anti-HBcAg were performed with assays purchased from Abbott Laboratories. Two of the animals that were initially negative for all markers were vaccinated with the human HBV vaccine and seroconverted for anti-HBsAg. These animals were considered uninfected with regard to the estimations of the percentage of the animals exposed to HBV. Fourteen of the 30 (46.7%) animals were positive for at least one marker of HBV infection (Table 1), and this included animals in three of the six species of animals examined. Seven of the animals (23.3%) were PCR positive, and all of those tested ( $n = 6$ ) were positive for HBsAg, indicating that these animals were chronically infected with HBV. All chronic carriers were members of either the *Hylobates agilis* or *Hylobates moloch* species. Eight of the animals (26.7%) were positive for antibodies to HBsAg, suggesting viral clearance; however, one of the anti-HBsAg-reactive animals was a chronic carrier (Ling [*H. moloch*]). Thirteen of the animals (43.3%) were positive for anti-HBcAg, and all anti-HBcAg-positive animals were positive for either HBsAg or anti-HBsAg, demonstrating that the anti-HBcAg positivity was not due to false reactivity. Thus, a high percentage of gibbons at this facility had prior exposure to a hepadnavirus, and 50% of the exposed animals were chronically infected.

Examination of the family tree of the *H. agilis* species suggested a pattern of vertical transmission resulting in chronic infection and horizontal transmission resulting in viral clearance. Of the four chronically infected *H. agilis* animals, Pepino, Phoebe, and Homer shared a common sire that was negative for HBV markers and a dam that was not available for testing, but who was most likely a carrier that resulted in maternal transmission to all three offspring. The other animal with chronic infection among the *H. agilis* group, Felix, was the offspring of Pepino and Phoebe. Mumma, an anti-HBsAg-, anti-HBcAg-positive dam, mated with Pepino to produce Albert, who possessed no markers of HBV infection. Mumma's offspring by another sire was also negative for all HBV markers. A similar pattern was noted among the *H. moloch* animals. Two of the chronically infected animals, Chloe and Chilibi, were brother and sister, suggesting transmission from the mother. Shelby, who was first housed with Chloe as an adult, was anti-HBsAg, anti-HBcAg positive. Another chronically infected animal, Ling, had no exposure to Chloe or Chilibi. The antibody-positive status of Ushko could be attributed to being housed first with the sister of Chloe and Chilibi (who was

\* Corresponding author. Mailing address: Department of Virology and Immunology, Southwest Regional Primate Research Center, Southwest Foundation for Biomedical Research, 7620 N.W. Loop 410, San Antonio, TX 78227. Phone: (210) 258-9445. Fax: (210) 670-3329. E-mail: rlanford@icar.usfbr.org.

TABLE 1. HBV status of the gibbons examined in this study<sup>a</sup>

Gibbon <sup>b</sup>	Result by <sup>c</sup> :			
	PCR	HBsAg	Anti-HBsAg	Anti-HBcAg
<i>Hylobates agilis</i>				
Pepino (S)	+	+	-	+
Phoebe (D)	+	+	-	+
Felix (O)	+	ND <sup>d</sup>	-	ND
Homer	+	+	-	+
Sonny (S)	-	-	-	-
Elaine (D)	-	-	+	+
Betsy (O)	-	-	-	-
Mumma (D)	-	-	+	+
Albert (O)	-	-	-	-
Shorty (O)	-	-	+	+
<i>Hylobates moloch</i>				
Chloe	+	+	-	+
Shelby	-	-	+	+
Chilibi	+	+	-	+
Ling	+	+	+	+
Ivan	-	-	+	+
Ushko	-	-	+	+
<i>Hylobates pileatus</i>				
Birute (S)	-	-	+	+
JR (D)	-	-	-	-
Mat (O)	-	-	-	-
Cambio	-	-	-	-
Koko	-	-	-	-
<i>Hylobates syndactylus</i>				
Kino (S)	-	-	-	-
Fatima (D)	-	-	-	-
Ella (O)	-	-	-	-
Benny (O)	-	-	-	-
<i>Hylobates leucogenys</i>				
Ricky	-	-	-	-
Vok	-	-	-	-
<i>Hylobates gabriella</i>				
Kimkhi	-	-	-	-

<sup>a</sup> The following relationships are not indicated in the table. Sonny is the son of Mumma. Ushko was temporarily caged with both Ling (2 years) and Ivan (5 years). Cambio is the brother of JR. Koko is the son of Birute by a dam other than JR.

<sup>b</sup> S, sire; D, dam; O, offspring.

<sup>c</sup> PCR positivity was determined as described previously (5, 6). DNA was extracted from 10 µl of serum by proteinase K digestion and phenol-chloroform extraction. Primers were from the core region and were conserved among all HBV genotypes (forward primer, 5'-CCTTGGGTGGCTTTGGGGCA-3'; reverse primer, 5'-GGGCATTTGGTGGTCTATA-3'). Amplification was conducted for 35 cycles, and the products were detected by agarose gel electrophoresis and Southern hybridization. HBsAg, anti-HBsAg, and anti-HBcAg were determined by ELISA with assays purchased from Abbott Laboratories.

<sup>d</sup> ND, not determined.

chronically infected) and later with Ling. Thus, chronic HBV infections were found in three independent families. No overt sign of liver disease or mortality due to liver disease was noted in the chronically infected animals; however, since the animals were housed in a sanctuary, liver biopsies were not available for evaluation.

To examine the relationship of the viruses from gibbons to human HBV isolates, the core and surface genes were PCR amplified from serum-derived virion DNA and sequenced directly from the PCR products. The amino acid sequences from the gibbons were aligned with three human isolates of different genotypes: ayw3/France/genotype D, adw2/USA/genotype A, and adw4/Colombia/genotype F. Also included in the alignments were the sequences from the WMHBV isolate and the previously characterized gibbon sequence (Gibb1) (10). The sequences of all isolates are shown in reference to the consensus sequence. The sequences from Pepino, Phoebe, and Homer were identical for both genes, as were the sequences from Chloe and Chilibi, so only the sequences from Pepino and Chilibi are shown in the alignment. The core antigen amino acid sequences (Fig. 1) were similar for all isolates. The most notable difference was the 2-amino-acid insertion in the adw2 isolate at the same position as a size polymorphism in the WMHBV sequence (3-amino-acid deletion with respect to adw2, 1-amino-acid deletion with respect to all other isolates). Genotype F is the most divergent of the human genotypes (1), and the representative isolate of this genotype had unique amino acids in three positions not found in other isolates. In contrast, the WMHBV core gene contained unique amino acids in 14 positions. The core sequences of the gibbon isolates were very similar to those of the human isolates. Pepino contained unique residues at two positions and shared with Chilibi and Ling a single residue (asparagine 91) not found in the human isolates examined; however, this could not be considered a signature sequence for gibbon isolates, since it was lacking in the Gibb1 sequence. Pepino differed from Chilibi and Ling at two residues (amino acids 67 and 174), while Chilibi and Ling differed from each other by a single residue (amino acid 49).

The surface gene amino acid sequences (Fig. 2) were more divergent than the core genes; however, the gibbon isolates were still very similar to the human isolates. The WMHBV sequence has a 2-amino-acid insertion not seen in any of the other isolates. The genotype F isolate contained unique amino acids at 16 positions, while the WMHBV isolate differed from all other isolates at 28 positions. In contrast, the sequences of isolates from Pepino, Chilibi, and Ling were unique at one, four, and three positions, respectively. No signature changes were present in the sequences of the different gibbon isolates. Pepino differed from Chilibi at 12 positions, while Chilibi differed from Ling at 8 positions.

Phylogenetic analysis was performed to evaluate the evolutionary relationship of the various primate isolates of HBV. Analyses were performed with the PAUP\* program, to compare the nucleotide sequences (approximately 549 to 555 bp) of the core gene, as implemented previously (5). Although the surface gene is larger and generally more variable than the core gene, the core gene sequences generated a more robust phylogenetic tree (only one tree versus four different versions when surface gene sequences were used) and contained a greater number of parsimony-informative characters (118 versus 100). An exhaustive search for the most parsimonious trees for the core gene revealed a single tree (Fig. 3). The nucleotide sequences of the core genes of Pepino, Chilibi, and Ling were compared along with those of the previously reported isolates from a chimpanzee and a gibbon. Five human virus isolates



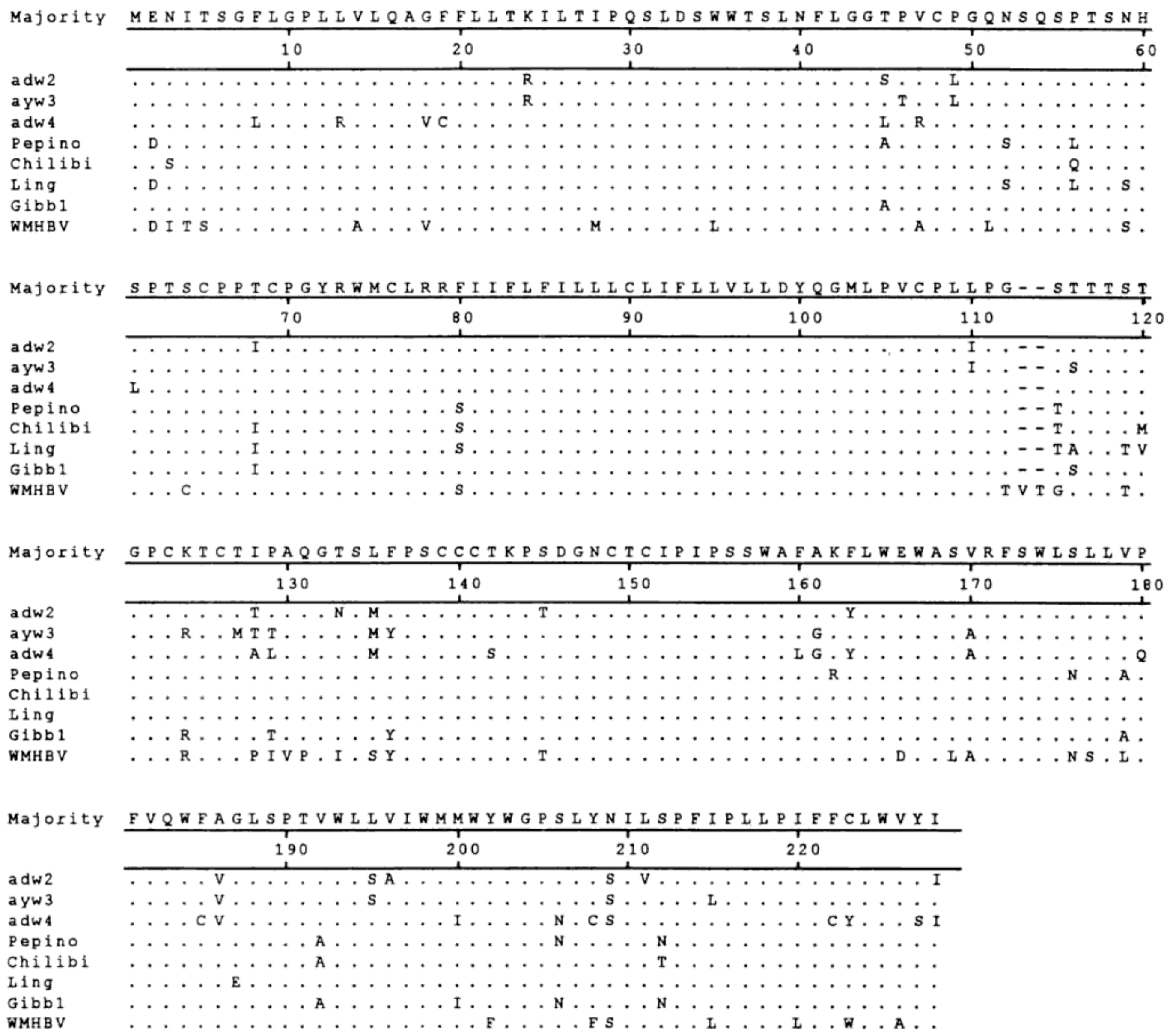


FIG. 2. Alignment of surface gene amino acid sequences from primate hepadnaviruses. The surface gene sequences from three human and four gibbon hepadnaviruses and one woolly monkey hepadnavirus were aligned as described in the legend to Fig. 1. All sequences are compared to the consensus sequence, with dots indicating identity and dashes indicating deletions. GenBank accession numbers are given in the legend to Fig. 3.

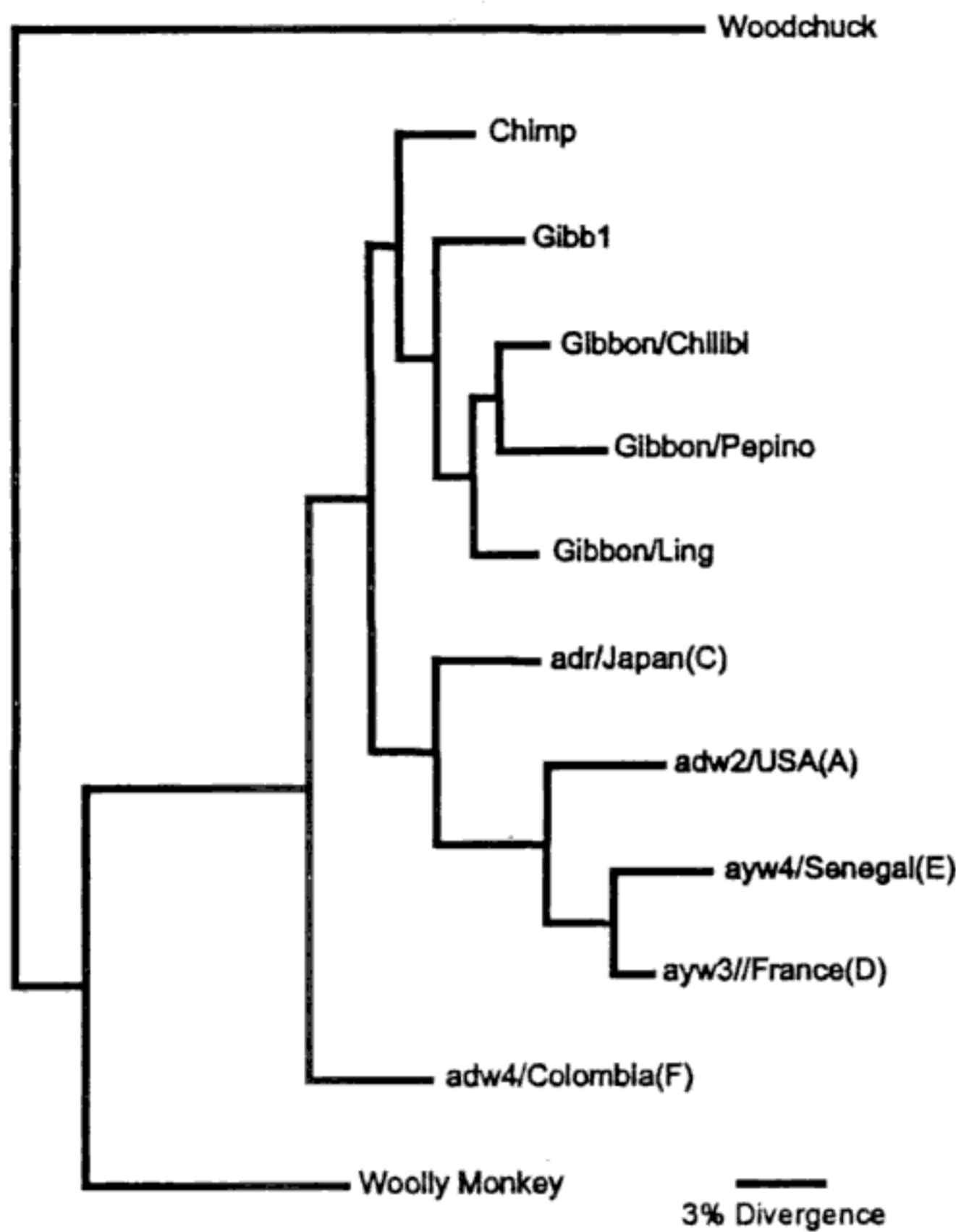


FIG. 3. Phylogenetic analyses of the nucleotide sequence of core genes from primate hepadnaviruses. Phylogenetic analyses were performed with the test version, 4.0b2, of the PAUP\* program written by David Swofford (Sinauer Associates, Sunderland, Mass.). Exhaustive searches made the use of statistical support for trees (e.g., BOOTSTRAP) unnecessary and gave the single tree shown here. The nucleotide sequences of the core antigen genes from gibbons Pepino, Chilibi, and Ling were compared with the previously reported isolates from a chimpanzee (Chimp) (14) and a gibbon (Gibb1) (10). Five human isolates representative of five of the six human genotypes were included with the genotype given in parentheses. The sequence of WMHBV (5) was included as an example of a primate HBV sequence outside of the human HBV family. A woodchuck isolate was used as the outgroup to root the trees. The distance along the horizontal axis among isolates is proportional to genetic divergence. The GenBank accession numbers for the sequences of the isolates shown here are as follows: adw2/USA (A), X02763; ayw4/Senegal(E), X75664; ayw3/France(D), V01460; adr/Japan(C), L08805; adw4/Colombia(F), X75663; Gibb1, U46935; Chimp, D00220; Woolly Monkey, AF046996; Woodchuck, J02442; Gibbon/Chilibi/Core, AF213008; Gibbon/Chilibi/Surface, AF213006; Gibbon/Pepino/Core, AF213010; Gibbon/Pepino/Surface, AF213007; Gibbon/Ling/Core, AF213009; Gibbon/Ling/Surface, AF213005.

tionships of the animals suggest this could be accounted for by vertical transmission from three chronically infected dams. The other seven HBV-exposed animals had cleared their infections and thus were more likely to have been exposed as adults.

Although the animals housed at the International Center for Gibbon Studies have not participated in medical experimentation, the wild-born animals were often captive pets of people in the area before being acquired by a refuge. Close contact with infected humans could easily account for the exposures, with gibbons being exposed to contaminated blood through biting

and/or scratching of infected humans, perhaps at the time of capture. A serological analysis of a large population of gibbons living in the wild was not feasible, leaving open the question of whether hepadnavirus infections persist in gibbons in the absence of current contact with humans. The transmission of viruses between nonhuman primates and humans can have dire consequences, as is expected to be the case for human immunodeficiency virus (3) and Ebola virus (2, 4). The transmission of human HBV to gibbons does not appear to have resulted in more severe infections in gibbons than those observed in humans. As the habitat of nonhuman primates continues to diminish, contact between humans and our closest relatives will increase, as will the cross-species transmission of viruses unique to each species. The outcomes cannot be predicted and may be benign or catastrophic. A continued surveillance of both humans and nonhuman primates for such occurrences is warranted.

This work was supported by grants CA53246 (R.E.L.), AI01124 (R.R.-H.), and P51 RR13986 from the National Institutes of Health.

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