Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes

Jan Felix Drexler[a,1], Andreas Geipel[b,1], Alexander König[b], Victor M. Corman[a], Debby van Riel[c], Lonneke M. Leijten[c], Corinna M. Bremer[b], Andrea Rasche[a], Veronika M. Cottontail[d,e], Gael D. Maganga[f], Mathias Schlegel[g], Marcel A. Müller[a], Alexander Adam[h], Stefan M. Klose[d], Aroldo José Borges Carneiro[i], Andreas Stöcker[j], Carlos Roberto Franke[i], Florian Gloza-Rausch[a,k], Joachim Geyer[l], Augustina Annan[m], Yaw Adu-Sarkodie[n], Samuel Oppong[n], Tabea Binger[a], Peter Vallo[d,o], Marco Tschapka[d,e], Rainer G. Ulrich[g], Wolfram H. Gerlich[b], Eric Leroy[f,p], Thijs Kuiken[c], Dieter Glebe[b,1,2], and Christian Drosten[a,1,2]

[a]Institute of Virology, University of Bonn Medical Centre, 53127 Bonn, Germany; [b]Institute of Medical Virology, Justus Liebig University, 35392 Giessen, Germany; [c]Department of Viroscience, Erasmus Medical Center, 3000 CA, Rotterdam, The Netherlands; [d]Institute of Experimental Ecology, University of Ulm, 89069 Ulm, Germany; [e]Smithsonian Tropical Research Institute, Balboa Ancón, Republic of Panamá; [f]Centre International de Recherches Médicales de Franceville, BP 769 Franceville, Gabon; [g]Friedrich-Loeffler-Institut, Institute for Novel and Emerging Infectious Diseases, 17493 Greifswald-Insel Riems, Germany; [h]Institute of Pathology, University of Cologne Medical Centre, 50937 Cologne, Germany; [i]School of Veterinary Medicine, Federal University of Bahia, 40.170-110, Salvador, Brazil; [j]Infectious Diseases Research Laboratory, University Hospital Prof. Edgard Santos, Federal University of Bahia, 40.110-060, Salvador, Brazil; [k]Noctalis, Centre for Bat Protection and Information, 23795 Bad Segeberg, Germany; [l]Institute of Pharmacology and Toxicology, Justus Liebig University, Biomedical Research Center, 35392 Giessen, Germany; [m]Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Kumasi, Ghana; [n]Faculty of Renewable Natural Resources,Kwame Nkrumah University of Science and Technology, Kumasi, Ghana; [o]Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, 60 365 Brno, Czech Republic; and [p]Institut de Recherche pour le Développement (IRD), Unité Mixte de Recherche 224 (MIVEGEC), IRD/Centre National de la Recherche Scientifique/Universite Montpellier 1, 34032 Montpellier, France

Edited by Robert A. Lamb, Northwestern University, Evanston, IL, and approved August 2, 2013 (received for review April 29, 2013)

The hepatitis B virus (HBV), family *Hepadnaviridae*, is one of most relevant human pathogens. HBV origins are enigmatic, and no zoonotic reservoirs are known. Here, we screened 3,080 specimens from 54 bat species representing 11 bat families for hepadnaviral DNA. Ten specimens (0.3%) from Panama and Gabon yielded unique hepadnaviruses in coancestral relation to HBV. Full genome sequencing allowed classification as three putative orthohepadnavirus species based on genome lengths (3,149–3,377 nt), presence of middle HBV surface and X-protein genes, and sequence distance criteria. Hepatic tropism in bats was shown by quantitative PCR and in situ hybridization. Infected livers showed histopathologic changes compatible with hepatitis. Human hepatocytes transfected with all three bat viruses cross-reacted with sera against the HBV core protein, concordant with the phylogenetic relatedness of these hepadnaviruses and HBV. One virus from *Uroderma bilobatum*, the tent-making bat, cross-reacted with monoclonal antibodies against the HBV antigenicity determining S domain. Up to 18.4% of bat sera contained antibodies against bat hepadnaviruses. Infectious clones were generated to study all three viruses in detail. Hepatitis D virus particles pseudotyped with surface proteins of *U. bilobatum* HBV, but neither of the other two viruses could infect primary human and *Tupaia belangeri* hepatocytes. Hepatocyte infection occurred through the human HBV receptor sodium taurocholate cotransporting polypeptide but could not be neutralized by sera from vaccinated humans. Antihepadnaviral treatment using an approved reverse transcriptase inhibitor blocked replication of all bat hepadnaviruses. Our data suggest that bats may have been ancestral sources of primate hepadnaviruses. The observed zoonotic potential might affect concepts aimed at eradicating HBV.

evolution | zoonosis | virome | metagenomics | reverse genetics

More than 40% of the human population has been infected with the hepatitis B virus (HBV), giving rise to 240 million chronic HBV carriers and *ca.* 620,000 HBV-associated deaths annually (1). A prophylactic vaccine containing the small HBV genotype A2 surface antigen (SHB) is part of the worldwide Expanded Program on Immunization. Because of the general success of SHBs-based vaccination, global eradication of HBV has been considered achievable (2, 3). Potential for the virus to be eradicated is supported by the fact that there are no known animal reservoirs. However, recent studies addressing the distribution of pathogens related to human viruses in wild animals, including mumps- and measles-related viruses in bats, have uncovered surprising putative novel reservoirs for human-pathogenic viruses (4).

Significance

Hepatitis B virus (HBV) is the prototype hepadnavirus; 40% of humans have current or past infection. In a global investigation of viral diversity in bats, we discovered three unique hepadnavirus species. The relatedness of these viruses to HBV suggests that bats might constitute ancestral sources of primate hepadnaviruses. Infection patterns in bats resembled human infection with HBV. After resurrection from bat tissues, pseudotyped viruses carrying surface proteins of one bat hepadnavirus could infect human liver cells. HBV vaccination is probably not protective against these viruses, but viral replication could be blocked by a reverse transcriptase inhibitor used as an anti-HBV drug in humans. The potential of bat hepadnaviruses to infect humans should be considered in programs aimed at eradicating HBV.

Author contributions: J.F.D., D.G., and C.D. designed research; J.F.D., A.G., A.K., V.M. Corman, D.v.R., L.M.L., C.M.B., A.R., M.A.M., A. Adam, A.J.B.C., A.S., and T.K. performed research; V.M. Cottontail, G.D.M., M.S., S.M.K., C.R.F., F.G.-R., J.G., A. Annan, Y.A.-S., S.O., T.B., P.V., M.T., R.G.U., W.H.G., E.L., and T.K. contributed new reagents/analytic tools; J.F.D., A.G., A.K., V.M. Corman, D.v.R., L.M.L., C.M.B., T.K., and D.G. analyzed data; J.F.D., D.G., and C.D. wrote the paper; V.M. Cottontail performed field work (Panama); G.D.M., P.V., and E.L. performed field work (Gabon); S.M.K. performed field work (Australia/Papua New Guinea); A.J.B.C. and C.R.F. performed field work (Brazil); F.G.-R. performed field work (Germany); and A. Annan, Y.A.-S., S.O., and T.B. performed field work (Ghana).

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper has been deposited in the GenBank database (accession nos. KC790373–KC790381).

[1]J.F.D., A.G., D.G., and C.D. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: dieter.glebe@viro.med.uni-giessen.de or drosten@virology-bonn.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1308049110/-/DCSupplemental.

MICROBIOLOGY

HBV is the prototype species of the family *Hepadnaviridae*, which comprises two genera: the genus *Orthohepadnavirus* associated with mammals and the genus *Avihepadnavirus* associated with birds. Phylogenetic studies suggested the presence of HBV in humans for at least 15,000 y (5). Recent analyses of avihepadnaviral sequences integrated into the genomes of several avian species suggested a much older origin, dating back at least 19 million y (6). No HBV genomic elements have so far been found in humans or other primates, preventing more precise estimates of the origins of primate HBV (i.e., human and nonhuman primate viruses). HBV strains can be divided into nine strictly human-associated genotypes (A–I). Additional strains outside some of those human-specific clades are known in chimpanzees, gorillas, gibbons, and orangutans (7). With sporadic exceptions (8), these primate HBV strains do not infect humans. The closest relative to human or ape viruses has been found in captive woolly monkeys (*Lagothrix lagotricha*), a South American nonhuman primate species (9). There are only three nonprimate orthohepadnaviruses, all being even less closely related to HBV. These viruses include woodchuck HBV from Eastern woodchucks (*Marmota monax*), Californian ground squirrel (*Otospermophilus beecheyi*) HBV, and arctic squirrel (*Spermophilus parryi*) HBV (10). These rodent hosts are endemic in circumscribed areas of North America, and their viruses are highly host-specific and cannot infect human hepatocytes (11, 12).

Within the ∼5,500 known terrestrial species of mammals, about 20% are bats. Close relatives of pathogenic human viruses have been described in bats over the last years, including Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS)-related coronaviruses (CoV) as well as filoviruses, such as Ebola- and Marburgvirus (13, 14). Among the multiple factors that facilitate virus evolution within and transmission from bats are their longevity, migratory activity, large and dense roosting communities, and close social interaction (14). We have analyzed earlier the role of bats in the evolution of pathogenic viruses using very large globally and phylogenetically comprehensive samples of animals (4, 15). In this study, we detected highly diversified bat hepadnaviruses capable of infecting human hepatocytes through the HBV-specific human receptor but not neutralized by SHBs vaccine-induced antibodies.

Results

HBV Detection. Bats were sampled between 2002 and 2011 in Panama, Brazil, Gabon, Ghana, Germany, Papua New Guinea, and Australia (Fig. 1). These specimens represented 54 different species and 11 of 18 extant bat families (Table S1). Serum and liver specimens from 3,080 individual bats were individually tested using two broadly reactive and highly sensitive nested PCR assays. The sensitivities of these PCR assays at 95% probability of detection were 41.3 (95% confidence interval = 29.7–75.9) and 64.7 (95% confidence interval = 47.3–112.9) international units/mL blood (Fig. S1 and Table S2). Liver specimens only were available for all 199 bats sampled in Brazil, and all other 2,881 specimens were sera. In 10 of 3,080 specimens (0.3%; all sera), HBV-related sequences were detected. Positive specimens stemmed from three different bat species. Among New World bats, 5 of 54 (9.3%) *Uroderma bilobatum* specimens from Panama tested positive. Among Old World bats, 4 of 51 *Hipposideros cf. ruber* specimens (7.9%) and 1 of 16 *Rhinolophus alcyone* specimens (6.3%) from Gabon contained HBV-like sequences. Fig. 1 shows the distribution areas of these bat species in gray.

Genome Organization. Full virus genome sequences were determined from all positive specimens of *H. cf. ruber*, the single specimen of *R. alcyone*, and four specimens of *U. bilobatum* (GenBank accession nos. KC790373–KC790381). The bat viruses formed three different lineages on preliminary phylogenetic inspection. Viruses from *H. cf. ruber* were collectively termed roundleaf bat HBV (RBHBV), the virus from *R. alcyone* was designated horseshoe bat HBV (HBHBV), and viruses from *U. bilobatum* were collectively termed tent-making bat HBV (TBHBV). Virus designations were chosen according to the designation of other non-human hepadnaviruses based on the common names of their hosts [e.g., Woolly monkey HBV (WMHBV)], and they are detailed in Fig. S2*A*. The genome organization and size of the putative open reading frames (ORFs) were compared with all other known hepadnaviruses (Fig. S2*B*). RBHBV genomes comprised 3,368 nt, with a total of 12–18 (0.4–0.5%) nucleotide exchanges between each other. HBHBV comprised a genome of 3,377 nt. The TBHBV genomes comprised 3,149 nt, which varied by 4–74 (0.1–2.3%) nucleotide exchanges from each other. RBHBV and HBHBV diverged by 19% of their genomic nucleotide sequence from each other and 39% of their genomic nucleotide sequence from TBHBV. All bat viruses varied in their nucleotide sequences by at least 35% from sequences of any known hepadnavirus (Tables S3 and S4). As in all orthohepadnaviruses, the unique bat viruses contained four ORFs identifiable as the surface *(S)*, polymerase *(P)*, core *(C)*, and *X*-ORFs (Fig. S2*C*). The position of all ORFs in bat hepadnaviruses was similar to ORFs of the known members of the *Orthohepadnavirus* genus but clearly distinct from ORFs of duck hepatitis B virus, the prototype avihepadnavirus. The sizes of all predicted ORFs compared with homologs in prototype hepadnaviruses are shown in Fig. S2*B*. Details for the comparison of translated amino acid sequences of each predicted virus protein are provided in Tables S3 and S4. The surface (S) protein genes encoded in the large open reading frame of all newly discovered hepadnaviral genomes contained a *preS1*, a *preS2*, and the *S* domain. The *preS1* domain contained an *N*-myristoylation signal necessary for myristoylation at glycine-2 of *preS1*. Typical *N*-glycosylation sites within the *preS2* and *S* domains were conserved, similar to HBV. Within the predicted antigenic SHBs loop, all eight essential cysteins for viral assembly, secretion, and infectivity (16) were present. Other than ORF organization, HBV and the bat hepadnaviruses also shared a similar location of the direct repeat (DR) sequences DR1 and DR2 involved in genome replication. In addition, secondary structure prediction highlighted the structural similarities between HBV and the bat hepadnaviruses in their ε-loops, which serve as templates for the priming of reverse transcription of pregenomic RNA in all hepadnaviruses (Fig. S2*D*).

According to a Bayesian phylogenetic analysis based on full ortho- and avihepadnavirus genomes, bat hepadnaviruses clustered

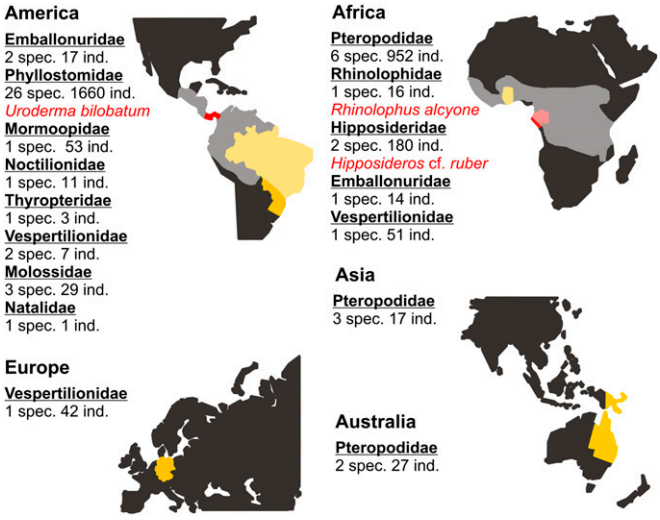

Fig. 1. Sampling sites and distribution of HBV-positive bat species. Sampling sites of HBV-positive bats are in red, and other sites are in yellow. Next to sites, the number of sampled species and specimens per family are given. Red, positive bat species; gray, distribution of positive bats.

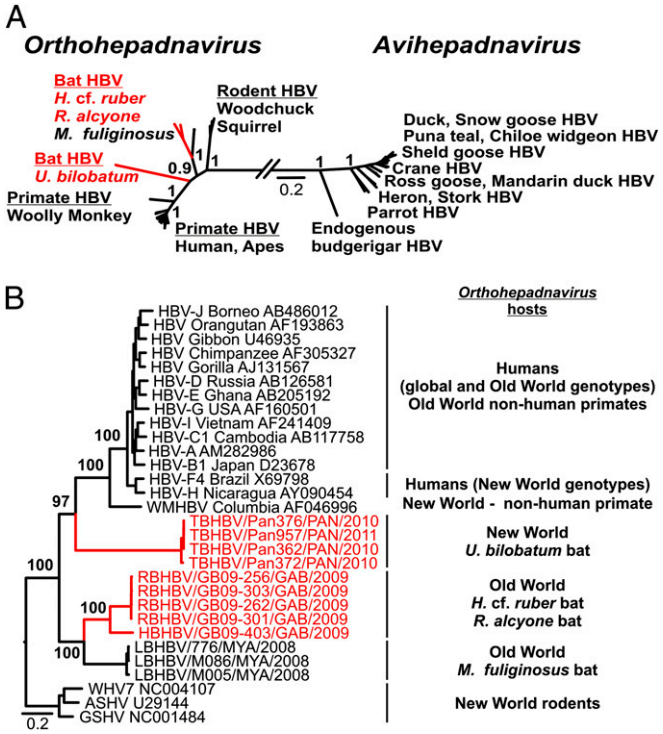

Fig. 2. Phylogenetic analysis including the unique bat viruses. (*A*) Bayesian phylogeny of *Hepadnaviridae* full genomes. The branch between ortho- and avihepadnaviruses was truncated for graphical reasons (interrupted lines). (*B*) ML full genome phylogeny. Unique bat viruses from this study are in red. Values at deep nodes represent Bayesian posterior probabilities or percentage ML bootstrap replicates; scale bars represent genetic distance.

within two highly supported monophyletic clades comprising the Old and New World bat viruses, respectively (Fig. 2*A*). The New World bat viruses formed a sister clade to all primate hepadnaviruses. Primate and New World bat viruses together were in sister relationship to all Old World bat viruses (RBHBV and HBHBV). Placement of the avihepadnaviruses as an outgroup to all orthohepadnaviruses resulted in a basal in-group bifurcation that separated the rodent viruses from all bat and primate viruses. A maximum likelihood (ML) analysis of the genus *Orthohepadnavirus* rooted accordingly yielded identical topologies with high statistical support (Fig. 2*B*). The recently described *Miniopterus* bat hepadnavirus from Myanmar (17) clustered as a sister clade to the African bat viruses (RBHBV and HBHBV) in both Bayesian and ML tree reconstructions.

Infection Patterns and Serological Relatedness to HBV. In all five bats from which solid organs were available, viral DNA in tissues was quantified by real-time PCR (Fig. 3*A*). Concentrations in liver tissue of the hipposiderid bats were significantly higher than concentrations in other organs or serum (*t* test, $P < 0.01$ for all). The highest virus concentration of 2.2×10^{12} copies/g was observed in liver tissue. In the single *R. alcyone* bat, high concentrations were also observed in lung tissue. For New World bats, no organ specimens were available. However, viremia was measured in the blood of all five *U. bilobatum*, yielding concentrations comparable with or higher than concentrations observed in Old World bats (median = 7.3×10^{10} copies/mL; range = 1.4×10^{6}–2.7×10^{11}).

Histopathology on available tissue samples showed few to moderate lymphocyte infiltrates and scant neutrophils/eosinophils in portal triads of hepadnavirus DNA-positive bats (Fig. 3*B*). In situ hybridization using an RNA probe directed against the full genome of RBHBV yielded clear staining patterns suggestive of deposits of viral DNA within the nuclei and cytoplasm of hepatocytes in an RBHBV DNA-positive animal; these patterns were not seen in control animals or with an irrelevant probe (Fig. 3*C* and Fig. S3*A*).

For additional investigation of gene and protein functions, full-length 1.1 infectious clones were generated for RBHBV, HBHBV, and TBHBV. For an assessment of serological reactivities in virus-infected bats, hepatoma cells were transfected with reconstructed RBHBV and HBHBV genome plasmids and stained with all available bat sera from the hepadnavirus DNA-positive species *H. cf. ruber* and *R. alcyone* in an immunofluorescence assay (IFA). Seroreactivities in New World bats were not assessed, because secondary antibodies against these species are not available. Antibody detection rates were high, with 18.4% in hipposiderid bats (9 of 49 animals) and 6.3% in rhinolophid bats (1 of 16 animals). Reactivity was observed in nuclei and cytoplasm, resembling typical reaction patterns of human sera directed against HBV core antigen (HBcAg) (Fig. 3*D* and Fig. S3 *B* and *C*). IFA end-point titers ranged from 1:100 to 1:1,600, with a median of 1:400 in hipposiderid bats. The rhinolophid bat showed both viral DNA and a high titer against the homologous HBHBV (1:1,600). Of nine antibody-positive hipposiderid bats, two bats showed concomitant viral DNA and antibodies against the homologous RBHBV, seven bats showed antibodies only, and two bats showed viral DNA only. Table S5 provides details on DNA and antibody detection rates.

To assess serologic cross-recognition of the African bat hepadnaviruses, all IFA-positive specimens were also tested for antibody reactivity with the heterologous virus. The antibody-positive serum from the rhinolophid bat cross-recognized the hipposiderid bat virus antigens, and six of nine antibody-positive hipposiderid bat sera cross-recognized the rhinolophid bat virus antigens. Those three sera that did not show cross-reactivity had lower titers than the other sera (below 1:400).

The existence of anti-*S* antibodies was evaluated using cells transfected with plasmids, allowing expression of the large HBV

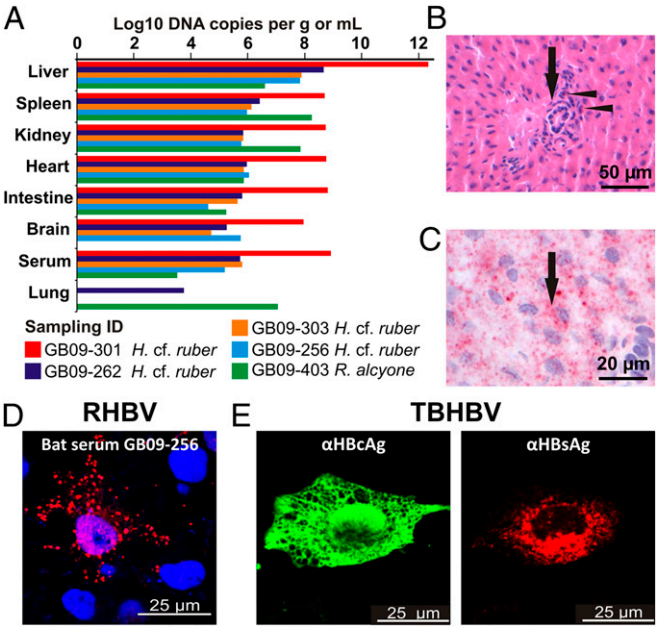

Fig. 3. Presentation of bat hepadnavirus infection. (*A*) Log10 hepadnavirus DNA copies per gram or milliliter. (*B*) H&E staining of hepadnavirus-positive hipposiderid bat GB09-301 liver. Arrow, lymphocytes; arrowheads, neutrophils and eosinophils. (*C*) In situ hybridization of hepadnavirus DNA in GB09-301 liver. (*D*) Immunofluorescence reaction pattern of hipposiderid bat serum GB09-256 with RBHBV-transfected HepG2 cells. (*E*) Reactivity of a polyclonal serum against the HBV core (green) and an mAb against an HBV surface epitope (red) in TBHBV-transfected HepG2 cells.

surface protein (LHBs) antigens only. Of 10 IFA-reactive bat sera, only 1 hipposiderid serum showed detectable anti-*S* antibodies. This serum also reacted weakly with the LHBs proteins of the heterologous rhinolophid bat virus. In summary, these results suggested infection patterns similar to patterns seen in human HBV.

Poly- and monoclonal anti-HBV antibodies (pAb and mAb) revealed a particularly close serological relatedness between New World bat and primate viruses, concordant with phylogeny. For these viruses only, there was cross-reactivity between their main surface antigen determinants (*S*-gene amino acids 121–124), whereas cross-reactivities between primate and Old World bat viruses extended only to core proteins (Fig. 3*E* and Fig. S3*C*).

To determine secretion of bat hepadnaviral surface proteins in the form of subviral particles (a hallmark of all *Hepadnaviridae*), the binding motif of the mAb HB1 reacting with a linear epitope of the antigenic determinant of the HBV surface proteins was used. To this end, the respective sites of the *S*-ORFs of RBHBV and HBHBV were substituted with the HB1 binding motif of the human genotype D by site-directed mutagenesis. In the case of TBHBV, a substitution was not necessary caused by the naturally occurring binding motif. The authentic *S* protein of TBHBV and the modified *S* proteins of RBHBV and HBHBV were secreted and detected by Western blotting in both the glycosylated and nonglycosylated forms (Fig. S3*D*).

Replicative Capability in Human Cells. Because phylogenetic and serologic results suggested a zoonotic potential of bat hepadnaviruses, the 1.1 overlength expression vectors were tested for their capability to replicate after transfection into human hepatoma HepG2 cells. Because hepadnaviruses replicate through a reverse transcription (RT) step, the ability of the nucleoside RT inhibitor Entecavir to inhibit viral replication and thus, formation and secretion of viral particles was tested. The reverse transcriptase activity of all bat hepadnaviral constructs was inhibited by Entecavir in a dose-dependent manner comparable with HBV (Fig. S4*A*). The IC_{50} of Entecavir was below 10 nM for HBV and all bat hepadnaviruses. Because RT of HBV requires interaction of the viral polymerase and encapsidated pregenomic mRNA (immature core particle), these data proved pregenomic mRNA transcription and translation of functional polymerase and core gene products of bat hepadnaviruses in human cells.

Potential to Infect Human Cells. The N-terminal LHBs amino acid residues encompassing the *preS1* domain are essential for infectivity of HBV. The unique bat hepadnaviruses showed high sequence identity within a short amino acid stretch (NPLGFFPDH) that is highly conserved within primate HBV, including WMHBV, but not rodent viruses, such as Woodchuck hepatitis virus (WHV) (Fig. S4*B*). Within adjacent accessory domains (residues NPDWD and NKDHWPEANKVGVG), only TBHBV, but not the Old Word bat viruses, showed high levels of sequence identity to primate HBV. For functional investigations, synthetic myristoylated (myr) *preS1* peptides derived from the three bat hepadnaviruses were tested for their potential to inhibit HBV infection of susceptible primary human hepatocytes (PHHs) and their widely used surrogate from *Tupaias*, *T. belangeri* primary hepatocyte (PTH). Inhibition of HBV infection was possible within nanomolar concentrations of all bat hepadnaviral myr-*preS1* peptides (Fig. 4). Myr-*preS1* peptides derived from Old World bat hepadnaviruses (HBHBV and RBHBV) showed decreased inhibitory potential in PHH, with IC_{50} values 50 to 60 times higher than values observed for TBHBV. An myr-*preS1* peptide from WHV was used as a control and showed no reactivity at all. In PTH, myr-*preS1* peptides from both HBHBV and TBHBV showed similar IC_{50} values (Fig. S4*C*). This observation correlated with the binding activity of these myr-*preS1* peptides to PTH (Fig. S4*D*). In summary, these data showed that myr-*preS1* peptides of bat hepadnaviruses were able to bind to HBV-susceptible hepatocytes, thereby inhibiting HBV infection in vitro.

To further analyze the zoonotic potential of bat hepadnaviruses, hepatitis-Δ virus (HDV) particles pseudotyped with surface proteins of all three bat hepadnaviruses were generated (Fig. S4*E*). However, only HDV pseudotyped with surface proteins of TBHBV (HDV_{TBHBV}) was able to infect both PTH and PHH, a pattern similar to the pattern observed with HDV_{HBV} (Fig. 5*A*). Specificity of infection was shown by the addition of infection-interfering myr-*preS1* peptides during HDV inoculation.

The infection-neutralizing potential of sera from persons successfully vaccinated against HBV with the standard HBV vaccine was assessed. Fig. 5*B* shows that two different human sera efficiently neutralized infection of PTH with HDV_{HBV} but not HDV_{TBHBV}. Addition of an mAb (HB1) directed against the antigenic loop of the *S* domain of HBV and TBHBV lowered infectivity in both cases. However, a strong, nearly complete neutralization was only achieved for HDV_{HBV}. Another mAb (C20/2), recognizing a conformational epitope in the antigenic loop within the *S* domain of HBsAg, could efficiently neutralize HDV_{HBV} but not HDV_{TBHBV}, compatible with lower amino acid sequence identity of TBHBV in this domain.

The human sodium taurocholate cotransporting polypeptide (hNTCP) is a high-affinity receptor for HBV and HDV. Both HDV_{HBV} and HDV_{TBHBV} used hNTCP for infection of HepG2 cells transiently expressing this receptor molecule (Fig. 5*C* and Fig. S4*F*). In both pseudotypes, the infection could be inhibited by specific myr-*preS1* peptides. These results showed that, similar to HBV, the surface proteins of TBHBV supported infection of PTH and PHH by using the hNTCP receptor.

Discussion

We describe highly diversified HBV-related viruses in Old and New World bats that establish three unique orthohepadnavirus species according to the established sequence distance criteria (18). All known primate, rodent, and avian hepadnaviruses can cause hepatitis in their hosts, with the possibility of symptom-free viremic carriage (19). We confirmed hepatic tropism and high viremia in hepadnavirus-infected bats as well as inflammatory leukocyte infiltrations typical for hepatitis. We have not been able so far to conduct longitudinal sampling of HBV-positive bat populations, precluding any insight in disease progression and transmission. Transovarial transmission is typical for avihepadnaviruses, whereas rodent hepadnaviruses are predominantly transmitted perinatally; primate hepadnaviruses are transmitted both perinatally and sexually. The extent of perinatal transmission could affect the extent of chronic virus carriage, which is a main characteristic of all hepadnaviruses (19, 20). We could not determine here the extent to which chronic infections occur in bats. High DNA detection rates speak in favor of prolonged courses of infection, but our finding of antibody-positive,

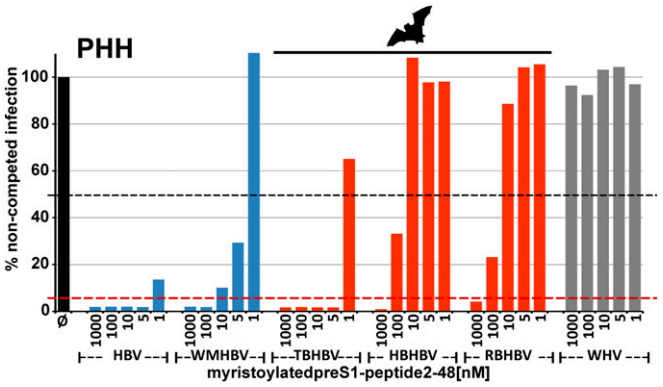

Fig. 4. Inhibition of HBV infection by competing *preS1* peptides. Newly synthesized and secreted HBsAg in supernatants of HBV-infected cultures 11–15 d postinfection of PHHs. Dashed red line, cutoff; dashed black line, IC_{50} value.

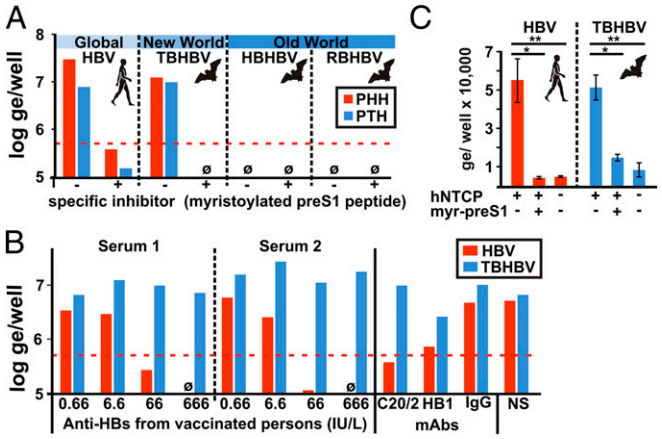

Fig. 5. Zoonotic potential of unique bat hepadnaviruses. (*A*) Infection with HDV_{HBV} or HDV_{TBHBV}. Presence (+) and absence (–) of specific myr-*preS1* peptide inhibitors. ge, HDV genome equivalent 12 d postinfection. (*B*) Lack of protection by antisera from HB-vaccinated persons. C20/2 and HB1, mAbs against the HBV surface; IgG, nonspecific mAb; NS, serum of a non-HepB–vaccinated person. (*C*) HBV and TBHBV use hNTCP for infection. HepG2 cells expressing hNTCP (+) or a control (–) were incubated with HDV_{HBV} or HDV_{TBHBV} with (+) or without (–) inhibitors. $*P < 0.05$, $**P < 0.02$; *t* test. Cutoff, dashed red line.

DNA-negative *Hipposideros* bats together with a high seroprevalence of 18.4% indicate that bats can probably clear the infection. Detection of DNA-positive, antibody-negative bats indicates sampling before seroconversion, whereas concomitant detection of DNA and antibodies indicates delayed clearance just like in humans. Also, the lack of detectable anti-LHBs antibodies in all but one bat resembles human disease, because these antibodies can be precipitated by high concentrations of HB antigen in serum.

The possibility of a bat origin of primate hepadnaviruses enables speculations regarding their evolution. Primate HBV has only been detected in one monophyletic ape taxon, the Hominoidea superfamily, as well as the rather distantly related Woolly monkey, a New World primate (7, 9). There is complete absence of detection in cercopithecoid monkeys (the Old World monkey sister clade to the Hominoidea) as well as lower nonsimiiform monkeys. This absence leaves doubts regarding virus–host cosegregation in primates and suggests a direct acquisition of HBV as a split-off from the stem lineage leading up to WMHBV by primates (21). It should be noted that WMHBV has never been redetected in wild or captive animals, and serological studies have failed to detect antibodies against HBV in other New World monkeys (7). WMHBV could, thus, have been acquired in captivity, and its actual host could have been either an Old or New World mammal. Of note, this animal could have been a bat, but the stem lineage leading up to primate viruses could also have been acquired from any other (probably mammalian) source.

Throughout Africa and to a much lesser extent, the Neotropical ecozone, the consumption of bats as wild game by humans is common practice (22). We have, therefore, performed in-depth studies of the zoonotic potential of the described bat hepadnaviruses using several well-established techniques. It has been shown that infectivity and host tropism of HBV is determined by highly conserved amino acids of the *preS1* domain and its myristic acid (19, 23). In contrast to rodent hepadnaviruses, all bat hepadnaviruses showed nearly complete conservation of this relevant domain, and its functional importance was reflected in the potential of myr-*preS1* peptides to compete with infection, similar to HBV in vitro and in vivo (24, 25). However, we observed marked differences between primary human (PHH) and *Tupaia* (PTH) hepatocyte cultures. Although both cell culture systems showed similar susceptibilities to human serum-derived HBV infection, PHH revealed that *preS1* peptides from TBHBV, but not HBHBV or RBHBV, reached IC_{50} values similar to HBV peptides. The inhibitory potential of myr-*preS1* peptides depends on their ability to block interaction of HBV with the newly discovered HBV receptor NTCP (26). Although the *Tupaia* and hNTCP sequences are very similar (26), our results suggest that PHH should be used in binding and entry studies to accurately evaluate the zoonotic potential of newly discovered hepadnaviruses.

Analysis of infection competence using HDV pseudotypes suggested that only TBHBV surface proteins were capable of mediating infection similar to HBV. The incompatibility of RBHBV and HBHBV proteins was remarkable given the clear ability of their *preS1* domains to bind hepatocytes and inhibit HBV infection. It should be mentioned that the work by Gudima et al. (27) reported infection of PHH, even with HDV particles pseudotyped with surface proteins from WHV (HDV_{WHV}), although WHV itself does not infect humans or chimpanzees. Gudima et al. (27) concluded that HDV_{WHV} must infect PHH using a different receptor, because *preS1* peptides from WHV, but not HBV, could inhibit HDV_{WHV} infection. In contrast to that study, we show that HDV_{TBHBV} and HDV_{HBV} could both be inhibited by *preS1* peptides of TBHBV and HBV with similar efficiencies. Furthermore, both HDV_{TBHBV} and HDV_{HBV} used the same HBV-specific receptor (hNTCP). Because HBHBV and RBHBV are replication-competent after transfection into human hepatoma cell lines, their restriction point might be the presence of a second (co-) receptor involved in HDV/HBV infection that might be highly species-specific. Characterization of the NTCP molecules of these two bat species might help elucidate whether HBHBV and RBHBV also use NTCP for hepatocyte entry.

Other than the *preS1* domain, the antigenic loop of the S domain is another independent determinant for HDV and HBV infectivity (16, 28). Among all bat hepadnaviruses, only TBHBV shared exactly the essential sequence C(R/K)TC within the antigenic loop with HBV and reacted with the mAb HB1 directed against this domain. These differences and others within the S domain might explain why TBHBV, but not the other bat hepadnaviruses, could infect PHH.

It should be mentioned that there are several limitations to our efforts to assess zoonotic potential. For instance, WMHBV can efficiently infect other New World primates but not Old World primates in vivo (29), whereas recombinant HDV_{WMHBV} viruses can infect human and chimpanzee primary hepatocytes (30). One might, therefore, argue that our surrogate assays may not properly reflect the in vivo situation. However, additional proof of zoonotic potential would require inoculation of chimpanzees, which is not ethical given the expected severity of infection. Severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator (uPA/SCID) mice engrafted with human hepatocytes could serve as alternative models (31), but they would have to be validated by comparisons with chimpanzees before application on TBHBV, which is still distantly related to HBV and thus, might behave differently in this model. Another limitation arises from the use of recombinant HDV/bat hepadnaviruses for infection and inhibition studies. However, because the recovery of homologous recombinant orthohepadnaviruses in sufficient amount is technically difficult, recombinant HDV/hepadnaviruses are widely accepted as surrogates (19, 27, 30).

In conclusion, among the three unique hepadnavirus species described in this report, we have evidence for a zoonotic potential for one of them, the New World bat-associated TBHBV. The lack of neutralization of this virus by high-titered anti-HBs sera from vaccinated individuals matches the observation of occasional failure of the standard vaccine, even against heterologous human HBV genotypes (32). Elimination of HBV from global circulation in humans is conceivable within several generations (3), but a revised vaccine formulation, including full LHBs or at least the critical *preS1* domain, could become

necessary to that end (33). Future vaccination concepts might also have to integrate considerations of the zoonotic potential of primate and nonprimate hepadnaviruses (2). It is unclear whether bat hepadnaviruses impose an ongoing risk of zoonotic human infections, but it should be considered that these viruses are genetically sufficiently distinct from HBV to go undetected in routine serological and molecular screening programs. Of note, access of humans to such HBV routine diagnostic programs cannot be deemed likely in the remote tropical areas from which these bats were sampled, highlighting the need for screening of human and nonhuman primate sera from these areas by broadly reactive diagnostic methods. Whereas ape populations are decreasing because of habitat exploitation on a global scale, bats can adapt to anthropogenic influence in multiple ways, leading to modifications of social structure, pathogen richness, and exposure to humans. We are only beginning to understand the role of bats as reservoirs of zoonotic viruses, emphasizing the importance of viral surveillance and the integration of ecological concepts into infectious disease epidemiology (34).

Materials and Methods

Sampling, Hepadnavirus Detection, and Characterization. Bats were caught as described previously (4). Permits are given in *SI Materials and Methods*. Purification, detection, and characterization of viral DNA were done as described previously and in *SI Materials and Methods* (4, 15, 35). Phylogenetic analyses were done using MrBayes (36) and PhyML (37).

HBV Constructs and Infection Experiments. Overlength constructs (1.1), surface expression vectors, and HDV pseudoparticles were generated as described previously (38–40). PHH and PTH infection, HBsAg detection, and infection of cells expressing the hNTCP were done as described before (41) and in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Monika Eschbach-Bludau, Sebastian Brünink, Tobias Bleicker, Thomas Kruppa, Mathieu Bourgarel, Vanessa Rüsseler, and Sigrun Broehl for technical assistance; Barbara Döring for the hNTCP-FLAG construct; Micha Nübling for HBV standards; and Aurelija Zvirbliene for mAb HB1. This study was funded by German Research Foundation (DFG) SPP 1596 Grants DR 810/1-1 (to J.F.D., M.T., and D.G.), GL 595/4-1 (to J.F.D., M.T., and D.G.), TS 81/6-1 (to J.F.D., M.T., and D.G.), DR 772/3-1 (to C.D.), and KA1241/18-1 (to M.T.); Brazilian Foundation for Research Support of the State of Bahia Grant SUS0038/2007 (to J.F.D. and C.R.F.); European Union FP7 Projects European Management Platform for Emerging and Re-Emerging Infectious Disease Entities Grant 223498; Europan Virus Archive Grant 228292 (to C.D.); Anticipating the Global Onset of Novel Epidemics Grant 278976 (to C.D.); and German Federal Ministry of Education and Research (BMBF) Grant 01KI1016D (to C.D.). The subproject in Panama was supported by the Smithsonian Tropical Research Institute and a personal scholarship of the German National Academic Foundation (to V.M. Cottontail).

1. Ott JJ, Stevens GA, Groeger J, Wiersma ST (2012) Global epidemiology of hepatitis B virus infection: New estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine* 30(12):2212–2219.
2. Chen DS (2010) Toward elimination and eradication of hepatitis B. *J Gastroenterol Hepatol* 25(1):19–25.
3. Alter HJ (2012) To have B or not to have B: Vaccine and the potential eradication of hepatitis B. *J Hepatol* 57(4):715–717.
4. Drexler JF, et al. (2012) Bats host major mammalian paramyxoviruses. *Nat Commun* 3:796.
5. Paraskevis D, et al. (2013) Dating the origin and dispersal of hepatitis B virus infection in humans and primates. *Hepatology* 57(3):908–916.
6. Gilbert C, Feschotte C (2010) Genomic fossils calibrate the long-term evolution of hepadnaviruses. *PLoS Biol* 8(9):e1000495.
7. Starkman SE, MacDonald DM, Lewis JC, Holmes EC, Simmonds P (2003) Geographic and species association of hepatitis B virus genotypes in non-human primates. *Virology* 314(1):381–393.
8. Tatematsu K, et al. (2009) A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. *J Virol* 83(20):10538–10547.
9. Lanford RE, Chavez D, Brasky KM, Burns RB, 3rd, Rico-Hesse R (1998) Isolation of a hepadnavirus from the woolly monkey, a New World primate. *Proc Natl Acad Sci USA* 95(10):5757–5761.
10. Simmonds P (2001) The origin and evolution of hepatitis viruses in humans. *J Gen Virol* 82(Pt 4):693–712.
11. Wang BJ, et al. (2011) Establishing a new animal model for hepadnaviral infection: Susceptibility of Chinese Marmota-species to woodchuck hepatitis virus infection. *J Gen Virol* 92(Pt 3):681–691.
12. Trueba D, et al. (1985) Transmission of ground squirrel hepatitis virus to homologous and heterologous hosts. *Hepatology* 5(3):435–439.
13. Annan A, et al. (2013) Human betacoronavirus 2c EMC/2012-related viruses in bats, Ghana and Europe. *Emerg Infect Dis* 19(3):456–459.
14. Luis AD, et al. (2013) A comparison of bats and rodents as reservoirs of zoonotic viruses: Are bats special? *Proc Biol Sci* 280(1756):20122753.
15. Drexler JF, et al. (2012) Bats worldwide carry hepatitis E virus-related viruses that form a putative novel genus within the family Hepeviridae. *J Virol* 86(17):9134–9147.
16. Salisse J, Sureau C (2009) A function essential to viral entry underlies the hepatitis B virus "a" determinant. *J Virol* 83(18):9321–9328.
17. He B, et al. (2013) Hepatitis virus in long-fingered bats, myanmar. *Emerg Infect Dis* 19(4):638–640.
18. Schaefer S (2007) Hepatitis B virus taxonomy and hepatitis B virus genotypes. *World J Gastroenterol* 13(1):14–21.
19. Glebe D, Urban S (2007) Viral and cellular determinants involved in hepadnaviral entry. *World J Gastroenterol* 13(1):22–38.
20. Liang TJ (2009) Hepatitis B: The virus and disease. *Hepatology* 49(5 Suppl):S13–S21.
21. Fares MA, Holmes EC (2002) A revised evolutionary history of hepatitis B virus (HBV). *J Mol Evol* 54(6):807–814.
22. Mickleburgh S, Waylen K, Racey P (2009) Bats as bushmeat: A global review. *Oryx* 43(2):217–234.
23. Glebe D (2006) Attachment sites and neutralising epitopes of hepatitis B virus. *Minerva Gastroenterol Dietol* 52(1):3–21.
24. Gripon P, Cannie I, Urban S (2005) Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein. *J Virol* 79(3):1613–1622.
25. Volz T, et al. (2013) The entry inhibitor Myrcludex-B efficiently blocks intrahepatic virus spreading in humanized mice previously infected with hepatitis B virus. *J Hepatol* 58(5):861–867.
26. Yan H, et al. (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 1:e00049.
27. Gudima S, et al. (2008) Primary human hepatocytes are susceptible to infection by hepatitis delta virus assembled with envelope proteins of woodchuck hepatitis virus. *J Virol* 82(15):7276–7283.
28. Le Duff Y, Blanchet M, Sureau C (2009) The pre-S1 and antigenic loop infectivity determinants of the hepatitis B virus envelope proteins are functionally independent. *J Virol* 83(23):12443–12451.
29. Lanford RE, Chavez D, Barrera A, Brasky KM (2003) An infectious clone of woolly monkey hepatitis B virus. *J Virol* 77(14):7814–7819.
30. Barrera A, Guerra B, Lee H, Lanford RE (2004) Analysis of host range phenotypes of primate hepadnaviruses by in vitro infections of hepatitis D virus pseudotypes. *J Virol* 78(10):5233–5243.
31. Sa-Nguanmoo P, et al. (2011) Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes. *Virus Res* 158(1–2):209–215.
32. Tacke F, et al. (2007) Acute hepatitis B virus infection by genotype F despite successful vaccination in an immune-competent German patient. *J Clin Virol* 38(4):353–357.
33. Niedre-Otomere B, et al. (2012) Recombinant Semliki Forest virus vectors encoding hepatitis B virus small surface and pre-S1 antigens induce broadly reactive neutralizing antibodies. *J Viral Hepat* 19(9):664–673.
34. Drosten C (2013) Virus ecology: A gap between detection and prediction. *Emerg Microbes Infect* 2:e31.
35. Drexler JF, et al. (2009) A novel diagnostic target in the hepatitis C virus genome. *PLoS Med* 6(2):e31.
36. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19(12):1572–1574.
37. Guindon S, et al. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Syst Biol* 59(3):307–321.
38. Nassal M (1992) The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. *J Virol* 66(7):4107–4116.
39. Glebe D, et al. (2005) Mapping of the hepatitis B virus attachment site by use of infection-inhibiting preS1 lipopeptides and tupaia hepatocytes. *Gastroenterology* 129(1):234–245.
40. Kuo MY, Chao M, Taylor J (1989) Initiation of replication of the human hepatitis delta virus genome from cloned DNA: Role of delta antigen. *J Virol* 63(5):1945–1950.
41. Glebe D, et al. (2003) Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. *J Virol* 77(17):9511–9521.

Supporting Information

Drexler et al. 10.1073/pnas.1308049110

SI Materials and Methods

Wildlife Permits and Ethics Clearances for Bat Sampling and Specimen Transfer. All animals were handled according to the European Union Council Directive 86/609/EEC for the protection of animals. Serum specimens were collected without killing any animals in Panama, Germany, Papua New Guinea, and Australia. In Brazil, Ghana, and Gabon, animals were dissected, and organ samples were additionally obtained under strict anesthesia of animals. Work in Brazil was supported by the Bahia state program for rabies control executed by ADAB (Agência Estadual de Defesa Agropecuária da Bahia). Individual permit numbers were Panama [Research-Permit STRI: STRI2563 (PI VC)-IACUC 100316–1001-18/Research-Permit ANAM: SE/A-68–11/Ethics-Permit: IACUC 100316–1001-18/Export Permits: SEX/A-30–11, SEX/A-55–11, SEX/A-81–10, SEX-A-26-10]; Ghana [Research Permit: 2008–2010 (A04957)/Ethics-Permit: CHRPE49/09/CITES/Export-Permit: State Agreement between Ghana and Hamburg (BNI)]; Australia [Research Permit: S11828 and S11762/Ethics-Permit: TRIM 01/1118(2), TRIM 06/3569, and University of Queensland/Animal Ethics Committee SIB600/05/DEST/Export-Permit: DE201-12]; Papua New Guinea (Ethics-Permit: PNG/NatMus/2002/Export-Permit: Conducted by Papua New Guinea National Museum); Gabon (Ethics-Permit: 00021/MEFEPA/SG/DGEF/DFC); Germany (Ethics-Permit: LANU 314/5327.74.1.6); and Brazil (Sampling permit IBAMA 15304–1; no specimens were exported).

Distribution of Hepadnavirus-Positive Bat Species. Distribution was adapted from the International Union for Conservation of Nature (1–3).

In Silico Analyses. Alignments were generated using MEGA5 (4). For MrBayes, 4 million generations were sampled every 100 steps, resulting in 40,000 trees; 25% of replicates were discarded as burn in. Maximum likelihood analyses used 1,000 bootstrap replicates. The HKY nucleotide substitution matrix was used for both. Statistical analyses were done using SPSS V20 (IBM).

Hepadnavirus Infectious Clones. For expression of hepadnaviruses in cell culture, the vector pCH-9/3091 was used that contains a 1.1 overlength hepatitis B virus (HBV) genome (genotype D) under the control of a human cytomegalovirus immediate early promoter (5) by substitution of pCH-9/3091 with an 1.1 overlength genome of the respective bat hepadnaviral genome [tent-making bat HBV (TBHBV), GenBank accession no. KC790379; horseshoe bat HBV (HBHBV), GenBank accession no. KC790377; roundleaf bat HBV (RBHBV), GenBank accession no. KC790374] (Fig. S2*A* shows nonabbreviated virus designations). Vectors for expression of surface proteins (large antigen open reading frame, L-ORF) under the control of natural hepadnaviral promoters contained a 5′-truncated version of the respective 1.1 overlength construct starting around 500 bp upstream of the corresponding L-ORF start codon (pCH9-3091-HBV-L, 506 bp; pCH9-TBHBV-L, 501 bp; pCH9-RBHBV-L, 519 bp; pCH9-HBHBV-L, 517 bp) in a pCH9-3091 backbone without human cytomegalovirus immediate early promoter. Insertion of the binding motif for mAb HB1 (amino acids 121–125 CRTCTT of HBV S domain) was done by substitution of the respective amino acids in the S domain of RBHBV (amino acids 119–124 CASCTI) and HBHBV (amino acids 119–124 CTSCTI) using site-directed mutagenesis.

Cell Lines and Transfection. Human hepatoma cell lines HuH7 and HepG2 were cultivated in DMEM with 10% (vol/vol) FCS (Invitrogen). Cells were transiently transfected with hepadnaviral 1.1 overlength expression plasmids at 80% confluence in 10-cm dishes using FuGene HD (Promega) according to the manufacturer's protocol; 1 d after transfection, cells were washed, and new medium was added. Cells and/or supernatants were used for assays 3 d posttransfection (p.t.) unless stated otherwise.

Production of Hepatitis-Δ Virus Pseudoparticles. For production of hepatitis-Δ virus (HDV) pseudoparticles, HuH7 cells were cotransfected with equimolar amounts of HDV plasmid pSVLD3. After transfection, cells were cultivated in DMEM/2% (vol/vol) FCS. Medium was changed every 3 d. Secreted HDV was quantified by determining genome equivalents in the supernatant by a specific RT-PCR at days 6 and 9. Supernatants from day 6 to day 9 were harvested at days 6, 7, 8, and 9 and used for infection assays after concentration using membrane ultrafiltration (Vivaspin, MWCO 10 000; GE Healthcare) according to the manufacturer's instructions.

SDS/PAGE and Immunoblotting. Original or concentrated (10×) supernatants from cells transfected with the respective surface protein expression vectors were pretreated with NuPAGE LDS Sample Buffer (Invitrogen) together with 8% (wt/vol) DTT and incubated at 70 °C for 10 min. Samples were analyzed on a 12% (vol/vol) precast polyacrylamide gel (Invitrogen). Immunoblotting was done as previously described with mAb HB1 [1 μg/μL in 3% (wt/vol) low-fat milk powder in PBS] as the primary antibody, donkey anti-mouse antibody conjugated with alkaline phosphatase (AP; 1:10,000 in 3% low-fat milk powder in PBS; Dianova) as secondary antibody, and Immobilon Western chemiluminescence AP Substrate (Merck Millipore).

Purification and Detection of Viral RNA/DNA from Cells. Purification of nucleic acids from supernatants of cells transfected with HBV/HDV expression plasmids was done using the High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's protocol. Purified RNA from transfections with HDV genome plasmid was additionally digested with RNase-Free DNase I (Thermo Scientific) for 1 h at 37 °C. HDV RNA from infected cells was purified using the Nucleospin 8 RNAII Kit (Macherey-Nagel) according to the manufacturer's instructions. Quantification of HDV genomes was done by a specific quantitative one-step RT-PCR (verso one-step RT-PCR Kit; Thermo Scientific) using the primers and probes listed in Table S2. Quantification of hepadnavirus DNA was done by specific discriminative SYBR green quantitative PCR using ABsolute qPCR SYBR Green-Mix (Thermo Scientific) and the virus-specific primers listed in Table S2.

Myristoylated Hepadnaviral *PreS1* Peptides. Chemical synthesis of carboxyterminally strep-tagged (amino acid WSHPQFEK), N-terminally myristoylated hepadnaviral *preS1* peptides spanning amino acid residues 2–48 (myr-$preS1_{2–48}$) was performed by Biosynthesis Inc.

Primary Human Hepatocytes. Primary human hepatocytes (PHHs) were obtained from Primacyt Cell Culture Technology GmbH.

HDV Pseudotype and HBV Infection Assays. For *Tupaias belangeri* primary hepatocyte (PTH) and PHH infections with cell culture-derived HDV pseudoparticles, cells were incubated for 16 h at day 3 postseeding with five genome equivalents per cell in the

presence of 4% (vol/vol) PEG. HepG2 cells transfected with an expression plasmid of human sodium taurocholate cotransporting polypeptide (hNTCP) or mock transfected were infected 3 d p. t. with 10 genome equivalents per cell for 16 h in the presence of 4% PEG. Cells of all infection experiments were washed three times with medium after virus inoculation and cultivated at 37 °C. Media were replaced, and supernatants were collected at days 1, 4, 7, 11, and 15 postinfection (p.i.) for primary hepatocytes and every 2 d until day 12 p.i. for HepG2 cells. Medium for cultivation of HepG2 cells was additionally supplemented with 1.5% (vol/vol) DMSO at day 1 p.i. As a control for infection specificity of HBV/HDV by blocking of viral attachment, cells were preincubated with 1 μM appropriate myr-*preS1* peptides for 30 min before addition of virus. The myr-*preS1* peptides remained during the whole viral inoculation time with the cells (16 h). The IC_{50} values of the different myr-*preS1* peptides for HBV infection were determined by peptide competition assay with respective myr-*preS1* peptides (myr-$preS1_{2–48}$) of HBV, Woolly monkey HBV (WMHBV), TBHBV, HBHBV, RBHBV, or Woodchuck hepatitis virus (WHV) (Fig. S2*A* shows virus abbreviations).

ELISA for HBV Surface Antigen Detection. Newly synthesized and secreted for HBV surface antigen (HBsAg) of HBV-infected cultures was quantified by an established in-house anti-HBsAg sandwich ELISA as described before (6). Values are denoted as relative percentage of untreated controls.

Immunofluorescence Assay. For immunofluorescence (IF) detection of specific antibodies directed against HBHBV proteins in the serum of bats, HuH7 cells were transfected with 1.1 overlength expression plasmids of HBHBV. For IF detection of core and surface protein expression of HBV, TBHBV, HBHBV, and RBHBV in the human hepatoma cell line HepG2, cells were transfected with the corresponding 1.1 overlength expression plasmids. For detection of surface proteins of HBHBV and RBHBV, cells were also cotransfected with expression plasmids for the L-ORF of respective bat hepadnaviruses with an integrated binding epitope for mAb HB1 within the S domain. For each IF analysis, cells were fixed 3 d p.t. or 6 d p.i. in 3% (wt/vol) paraformaldehyde, and membranes were permeabilized by incubation with 0.2% Triton × 100/PBS for 30 min at room temperature. Nonspecific epitopes were blocked with DMEM/10% (vol/vol) FCS. Cells were incubated for 2 h with specific monoclonal and polyclonal antibodies (mAb and pAb), diluted 1:50–1:200 in 0.1% FCS/DMEM at 37 °C, and washed extensively with PBS. Bat sera were diluted 1:200, incubated with cells transfected with respective hepadnaviral expression vectors, and detected with a goat anti-bat IgG secondary antibody (1:100 dilution; Bethyl) as described previously (7). After washing, cells were incubated with respective secondary antibodies conjugated with Alexa594 or Alexa488 dyes (Invitrogen). then, nuclei were stained with DAPI (Invitrogen), washed five times, and mounted on glass slides using Mowiol (Sigma-Aldrich).

In Situ Hybridization. RNAScope RNA probes were custom designed by Advanced Cell Diagnostics for HBHBV. In situ hybridization was performed as described by the manufacturer.

1. Sampaio E, et al. (2008) *Uroderma bilobatum. IUCN 2012. IUCN Red List of Threatened Species, version 2012.2*. Available at www.iucnredlist.org. Accessed March 14, 2013.
2. Mickleburgh S, Hutson AM, Bergmans W, Fahr J (2008) *Hipposideros ruber. IUCN 2012. IUCN Red List of Threatened Species, version 2012.2*. Available at www.iucnredlist.org. Accessed March 14, 2013.
3. Jacobs D, Cotterill FPD, Taylor PJ (2008) *Rhinolophus alcyone. IUCN 2012. IUCN Red List of Threatened Species, version 2012.2*. Available at www.iucnredlist.org. Accessed March 14, 2013.
4. Tamura K, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28(10):2731–2739.
5. Nassal M (1992) The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. *J Virol* 66(7):4107–4116.
6. Glebe D, et al. (2003) Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. *J Virol* 77(17):9511–9521.
7. Müller MA, et al. (2007) Coronavirus antibodies in African bat species. *Emerg Infect Dis* 13(9):1367–1370.

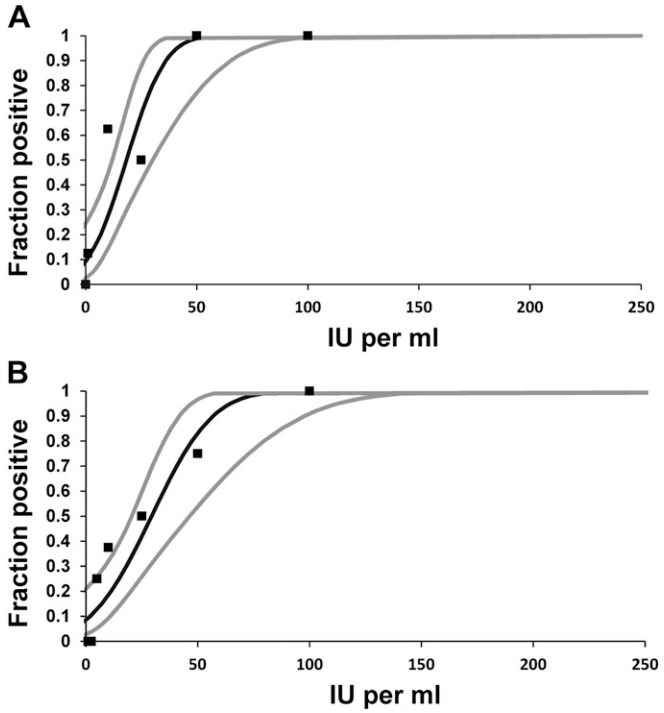

Fig. S1. Probit regression analyses of nested PCR assays used for hepadnavirus detection; 200 μL second World Health Organization international HBV reference standard containing 1 million international units (IUs) per milliliter (product code: 97/750; National Institute for Biological Standards and Control) were extracted using the MinElute Spin Kit (Qiagen) with an elution volume of 100 μL. The assay protocols and individual primer sequences are described in Table S2. *A* shows the first PCR assay, including primer HBV-F248. *B* shows the second assay, including primer HBVall-F1364, which allows detection of ortho- and avihepadnaviruses. Each plot depicts the observed proportion of positive results from eight parallel experiments at each dilution step as well as the derived predicted proportion of positive results at a given DNA input concentration. The black line shows the prediction, and the gray lines show the 95% confidence limits of the prediction.

A

Scientific host name	Distribution	Trivial host name	Name of associated hepadnavirus	Abbreviation
Homo sapiens, Hominidae	Global	Human, Apes	Hepatitis B virus	HBV
Marmota monax	New World	Eastern Woodchuck	Woodchuck hepatitis virus	WHV
Lagothrix lagotricha	New World	Brown Woolly monkey	Woolly monkey hepatitis B virus	WMHBV
Spermophilus parryi	New World	Arctic ground squirrel	Arctic ground squirrel hepatitis virus	ASHV
Otospermophilus beecheyi	New World	California ground squirrel	Ground squirrel hepatitis virus	GSHV
Uroderma bilobatum	New World	Tent-making bat	Tent-making bat hepatitis B virus	TBHBV
Rhinolophus alcyone	Old World	Horseshoe bat	Horseshoe bat hepatitis B virus	HBHBV
Hipposideros cf. *ruber*	Old World	Roundleaf bat	Roundleaf bat hepatitis B virus	RBHBV
Miniopterus fuliginosus	Old World	Long-fingered bat	Long-fingered bat hepatitis B virus	LBHBV

B

Species		Global								New World		Old World		New World	Global
		Human (genotypes)								Monkey		Bat		Wood-chuck	Duck
		A	B	C	D	E	F	G	H	WMHBV	TBHBV	HBHBV	RBHBV	WHV	DHBV
Genome length (nt)		3221	3215	3215	3182	3212	3215	3248	3215	3179	3149	3377	3368	3308	3027
Putative viral proteins (amino acids)															
HBc		185	183	183	183	183	183	195	183	182	188	189	189	188	262
PreC		29	29	29	29	29	29	Ø**	29	29	33	28	28	30	43
Surface proteins	LHBs	400	400	400	389	399	400	399	400	391	382	448	445	426	330
	SHBs	226	226	226	226	226	226	226	226	228	223	224	224	222	167
	PreS2	55	55	55	55	55	55	55	55	55	50	47	47	60	-
	PreS1	119	119	119	108	118	119	118	119	108	109	177	174	144	163*
Pol		845	843	843	832	842	843	842	843	835	827	902	899	879	788
HBx		154	154	154	154	154	154	154	154	152	135	141	141	141	-

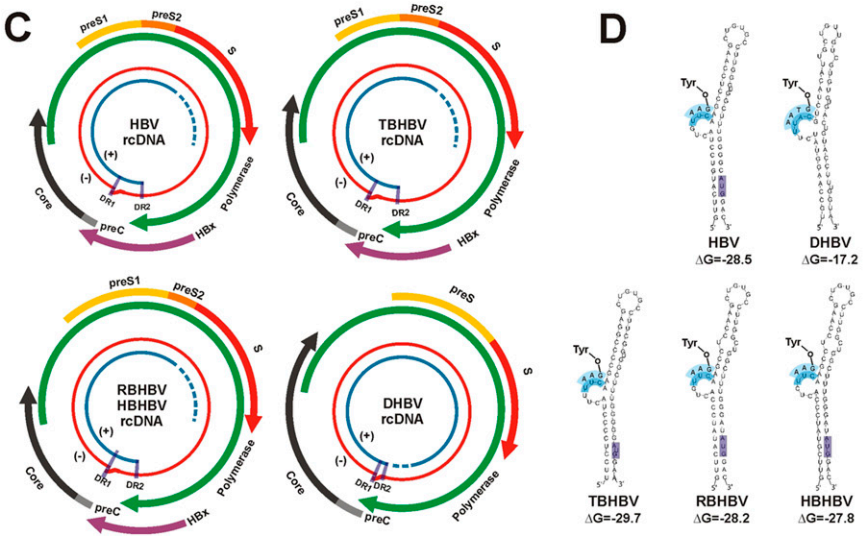

Fig. S2. Genomic features of newly discovered bat hepadnaviruses. *A* shows the abbreviations used for hepadnaviruses throughout the text in accordance with the current taxonomic proposals of the International Committee on Taxonomy of Viruses. *B* shows the sizes of the genomes and predicted ORFs of bat and prototype hepadnaviruses. *Duck hepatitis B virus (DHBV) as a typical representative of the genus *Avihepadnavirus* does not have a *preS1* or *preS2* domain but does have a preS domain and lacks the X-ORF. **Genotype G WT does not have an intact preC-ORF because of two stop codons. Human HBV genotypes have genomes of 3,182–3,248 nt. The genomes of WMHBV and TBHBV, both from hosts endemic in the New World, showed similar lengths of 3,179 and 3,149 nt, respectively. The two Old World viruses, RBHBV and HBHBV, had the largest genome lengths of all known hepadnaviruses, with 3,377–3,368 nt, because of an unusually large *preS1* domain-encoding sequence containing 177 and 174 codons, respectively. The genomes of these bat viruses were most similar to WHV, with a genome length of 3,308 nt and a *preS1* domain of 144 aa. *C* shows the genome organization of HBV (genotype D; GenBank accession no. AB126581), DHBV (GenBank accession no. HQ132730), RBHBV/HBHBV (shown together because of high similarity), and TBHBV. DR1/2, direct repeat 1/2; rcDNA, relaxed circular DNA; (+) and (–), orientation of DNA strands. The unknown length of the (+) strand in the unique bat hepadnaviruses is depicted in analogy to human HBV. The orthohepadnavirus S-ORF has the potential to encode three cocarboxyterminal surface proteins, putatively encoding large, medium, and small hepatitis B surface proteins. The S domain is present in all three surface proteins, whereas M contains an additional aminoterminal domain, termed *preS2*. The L protein contains *preS1*/*preS2* and the S domain. The P-ORF overlaps with the S- and C-ORFs, covering about two-thirds of the genome. The C-ORF contains a functional precore region encoding a signal peptide for optional secretion of the core protein, like in all WT hepadnaviruses. *D* shows RNA secondary structure predictions of the ε-structures of the respective sequences with S fold at 37 °C, 1 M NaCl, and no divalent cations (1). HBV and TBHBV ε-loops were very similar, whereas RBHBV and HBHBV differed in their loop structure by an additional unpaired region. Bat hepadnavirus and HBV ε-signals included the start codon (AUG) of the C-ORF (highlighted in pink) and showed the same nucleotide sequence in the unpaired region for the priming of the minus strand

Legend continued on following page

(UUC; highlighted in light blue). All secondary structures predicted for orthohepadnaviruses yielded similar free energy values ranging from 27.8 to 29.7 kcal/mol compared with 17.2 in DHBV. The predicted stability of the structures is shown in kilocalories per mol.

1. Chan CY, Lawrence CE, Ding Y (2005) Structure clustering features on the Sfold Web server. *Bioinformatics* 21(20):3926–3928.

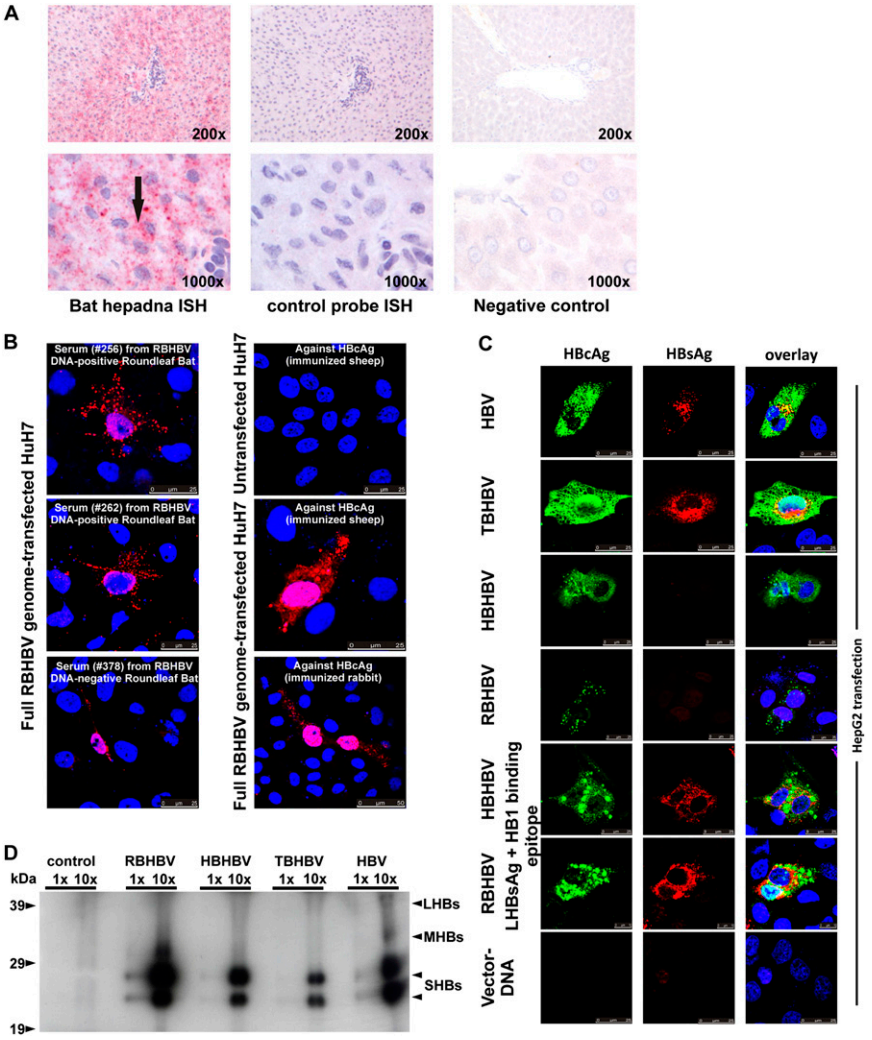

Fig. S3. Antigenic and histologic features of the unique bat hepadnaviruses. *A* shows detection of bat hepadnavirus DNA and mRNA by in situ hybridization (ISH) at a low and high magnification. (Magnification: *Upper*, 200×; *Lower*, 1,000×.) (*Left*) In a PCR-positive bat, a positive signal was observed in hepatocytes (arrow) surrounding the portal triads using hepadnavirus-specific probes. (*Center*) No signal was observed in serial tissue sections using an irrelevant control probe against a rodent hepacivirus (1). (*Right*) In a PCR-negative bat, no signal was observed using the hepadnavirus-specific probe. *B* shows (*Left*) recognition of *Hipposideros* bat hepadnavirus RBHBV antigens by three different *Hipposideros* bat sera and (*Right*) cross-recognition of RBHBV antigens by polyclonal control anti-HBc sera. *C* shows recognition of HBV, TBHBV, HBHBV, or RBHBV in transfected HepG2 cells by polyclonal anti-HBcAg (HBV core antigen) antibodies (green) and monoclonal anti-HBsAg antibody HB1 (red). For detection of HBHBsAg and RBHBsAg, expression plasmids for appropriate surface proteins with inserted HB1 binding epitope were cotransfected. *D* shows a Western blot with mAb HB1 reacting with a linear epitope of the antigenic determinant of the HBV surface proteins. For detection of RBHBV and HBHBV, the HB1 epitope of HBV genotype D was introduced in the corresponding region of the S-ORF. LHB, large HBV surface proteins; MHB, medium HBV surface proteins; SHB, small HBV surface proteins.

1. Drexler JF, et al. (2013) Evidence for novel hepaciviruses in rodents. *PLoS Pathog* 9(6):e1003438.

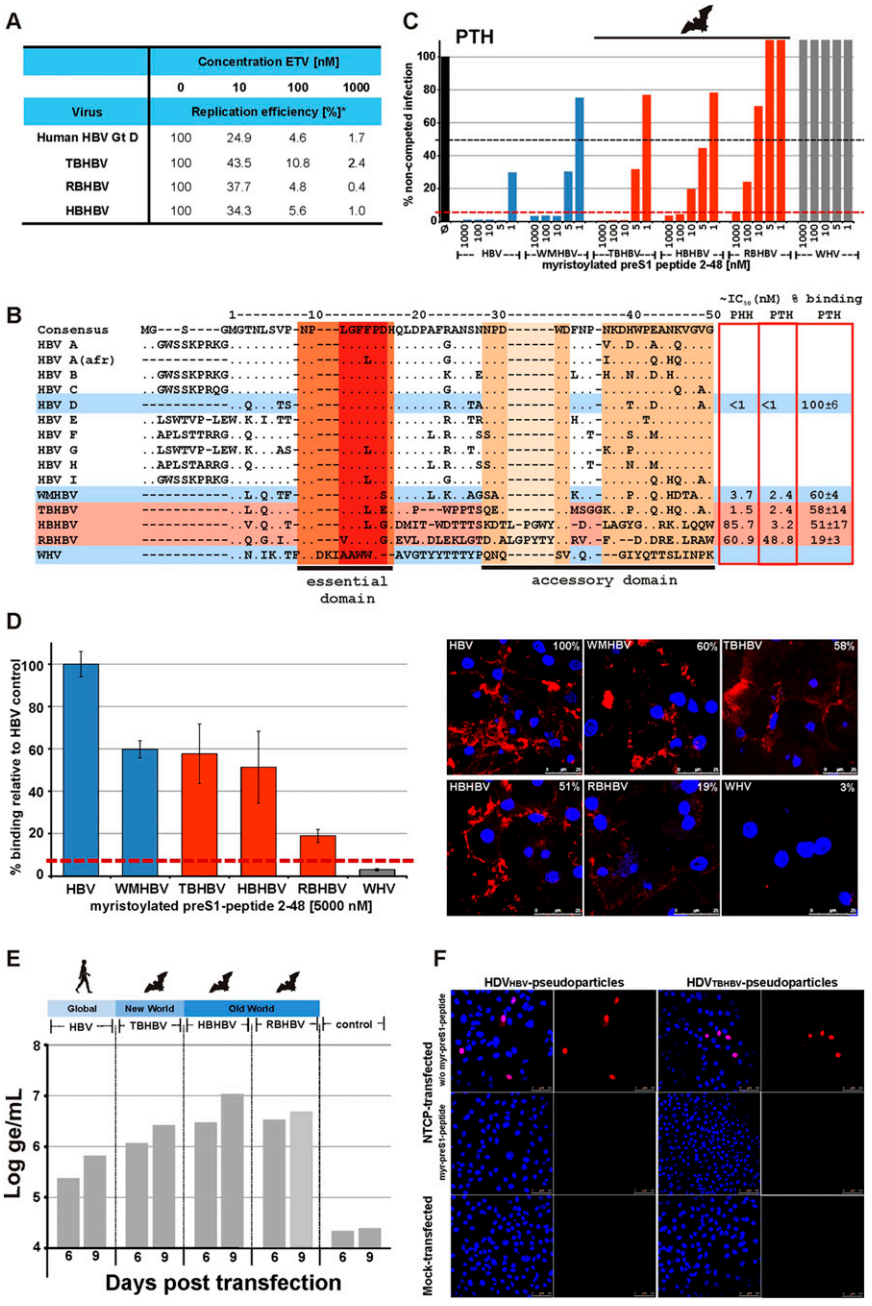

Fig. S4. Molecular features of bat hepadnaviruses. *A* shows the inhibition of bat hepadnavirus replication in hepatoma cells by the reverse transcriptase inhibitor Entecavir. *Replication was measured in the supernatant of cells 3 d p.t. by quantitative HBV PCR relative to control cells. *B* shows an alignment of the aminoterminal *preS1* sequence. Myr-*preS1* peptides amino acids 2–48 of indicated hepadnaviral species were used (red, bat hepadnaviruses). IC_{50} values were of the respective myr-*preS1* peptide on HBV infection using PTH or PHH. Binding, percent binding of the respective myr-*preS1* peptide to PTH. Essential and accessory domains of the HBV *preS1* domain important for virus–receptor interaction and inhibitory effect of myr-*preS1* peptides are indicated (1). *C* shows inhibition of HBV infection by competing *preS1* peptides of the different bat viruses: HBV, WMHBV, and WHV. Inhibition was analyzed by measuring newly synthesized and secreted HBsAg within supernatants of HBV-infected cultures from day 11 to day 15 p.i. from three experiments on PTH. Dashed red line, cutoff; dashed black line, IC_{50}. *D* shows binding of *preS1* peptides from different hepadnaviruses to PTH. Cells were precooled on ice and incubated with different strep-tagged myr-*preS1* peptides 2–48 (5,000 nM) from HBV, WMHBV, TBHBV, HBHBV, RBHBV, or WHV. (*Left*) After normalization to cell counts, relative binding potential of each peptide was adjusted to binding of human myr-*preS1* peptides. By using specific mouse antistrep antibody and Alexa594-conjugated anti-mouse antibodies, membrane binding was detected by confocal imaging (red). (*Right*) Alexa594 Pixel Sum of five overview pictures with 14–75 nuclei from each duplicate was detected by Leica LAS-AF software in three experiments. *E* shows the generation of pseudotyped HDV particles. The human hepatoma cell line HuH7 was cotransfected with plasmid pSVLD3 (for transcription of a complete replication-competent HDV genome) and expression plasmids for all three surface proteins of the respective hepadnaviruses or a control vector. Medium was changed every 3 d, and HDV genome equivalents of secreted pseudovirions were quantified at days 6 and 9 p.t. Supernatants taken on day 9 were concentrated and used for infection. All used orthohepadnaviral surface proteins showed the amino acid sequence responsible for interaction with Δ-antigen (l**W**MM**W**Y**W**), especially the three essential tryptophan residues. *F* shows staining of hepatitis-Δ antigen in HepG2 cells transfected with hNTCP and infected with HBV and TBHBV HDV pseudotypes. Cells were fixed at day 6 p.i. and stained for appearance of Δ-antigen (red). Cell nuclei were stained with DAPI (blue).

1. Glebe D, et al. (2005) Mapping of the hepatitis B virus attachment site by use of infection-inhibiting *preS1* lipopeptides and tupaia hepatocytes. *Gastroenterology* 129(1):234–245.

Table S1. Sample characteristics

Order-family and species	No. of samples	PCR positive (%)	Sampling site (y)
Chiroptera-Pteropodidae			
Dobsonia praedatrix	9		PNG (2002)
Eidolon helvum	348		GHA (2009, 2010)
Epomops franquetti	100		GAB (2009)
Hypsignathus monstrosus	100		GAB (2009)
Melonycteris melanops	7		PNG (2002)
Micropterus pusillus	100		GAB (2009)
Myonycteris torquata	100		GAB (2009)
Pteropus alecto	3		AUS (2006)
Pteropus poliocephalus	24		AUS (2006)
Rousettus aegyptiacus	204		GAB (2009)
Rousettus amplexicaudatus	1		PNG (2002)
Chiroptera-Emballonuridae			
Coleura afra	14		GAB (2009)
Saccopteryx bilineata	14		PAN (2010, 2011)
Saccopteryx leptura	3		PAN (2010, 2011)
Chiroptera-Hipposideridae			
Hipposideros cf. ruber	51	4 (7.9)	GAB (2009*)
Hipposideros gigas	129		GAB (2009)
Chiroptera-Molossidae			
Molossus currentium	10		BRA (2009)
Molossus rufus	17		BRA (2009)
Molossus molossus	2		BRA (2009), PAN (2010, 2011)
Chiroptera-Mormoopidae			
Pteronotus parnellii	53		PAN (2010, 2011)
Chiroptera-Natalidae			
Natalus stramineus	1		PAN (2010, 2011)
Chiroptera-Noctilionidae			
Noctilio leporinus	11		PAN (2005, 2010, 2011)
Chiroptera-Phyllostomidae			
Artibeus jamaicensis	981		PAN (2005, 2010, 2011)
Artibeus lituratus	94		PAN (2005, 2010, 2011)
Artibeus phaeotis	10		PAN (2010, 2011)
Artibeus watsoni	16		PAN (2010, 2011)
Carollia brevicauda	104		BRA (2009)
Carollia castanea	30		PAN (2010, 2011)
Carollia perspicillata	181		BRA (2009), PAN (2010, 2011)
Carollia spec.	15		BRA (2009)
Chiroderma villosum	2		PAN (2010, 2011)
Desmodus rotundus	31		BRA (2009), PAN (2010, 2011)
Glossophaga sorcina	27		BRA (2009), PAN (2010, 2011)
Lonchorhina aurita	1		BRA (2009)
Lophostoma brasiliense	4		PAN (2010, 2011)
Lophostoma silvicolum	17		PAN (2010, 2011)
Micronycteris hirsuta	3		PAN (2010, 2011)
Micronycteris microtis	9		PAN (2010, 2011)
Micronycteris minuta	1		PAN (2010, 2011)
Mimon crenulatum	4		PAN (2010, 2011)
Phylloderma stenops	5		PAN (2010, 2011)
Phyllostomus discolor	10		PAN (2010, 2011)
Phyllostomus hastatus	16		PAN (2010, 2011)
Platyrrhinus helleri	6		PAN (2010, 2011)
Tonatia saurophila	14		PAN (2010, 2011)
Trachops cirrhosus	18		BRA (2009), PAN (2010, 2011)
Uroderma bilobatum	54	5 (9.3)	PAN (2010*, 2011*)
Vampyressa pusilla	3		PAN (2010, 2011)
Vampyrodes caraccioli	4		PAN (2010, 2011)
Chiroptera-Rhinolophidae			
Rhinolophus alcyone	16	1 (6.3)	GAB (2009*)
Chiroptera-Thyropteridae			
Thyroptera tricolor	3		PAN (2010, 2011)
Chiroptera-Vespertilionidae			
Miniopterus inflatus	51		GAB (2009)

Table S1. Cont.

Order-family and species	No. of samples	PCR positive (%)	Sampling site (y)
Myotis daubentonii	42		GER (2009)
Myotis nigricans	5		PAN (2010, 2011)
Rhogeessa tumida	2		PAN (2010, 2011)
Total (11 families, 54 species)	3,080	10 (0.3)	

AUS, Australia; BRA, Brazil; GAB, Gabon; GER, Germany; PAN, Panama; PNG, Papua New Guinea.
*Positive sampling sites/years

Table S2. Oligonucleotides used for PCR screening and virus quantification

Genomic target region and oligonucleotide identity	Sequence (5′–3′)	Polarity	Assay used*
Surface/polymerase[†]			
HBV-F248	CTAGATTBGTGGTGGACTTCTCTCA	+	Screening PCR first/second round
HBV-R397	GATARAACGCCGCAGATACATCCA	–	Screening PCR second round
HBV-R450a	TCCAGGAGAACCAAYAAGAAAGTGA	–	Screening PCR first round
HBV-R450b	TCCAGGAGAACCAAYAAGAAGATGA	–	Screening PCR first round
Surface/polymerase[‡]			
HBVall-F1364	CTAGATTSGTGGTGGAYTTCTCTC	+	Screening PCR first round
HBVall-F1372	GTGGTGGAYTTCTCTCAGTTYTC	+	Screening PCR second round
HBVall-R1620a	GAGAAAMGGRCTGAGRCCSACTCCCAT	–	Screening PCR first round
HBVall-R1620b	GAGAAAMGGRGAGAGRCCSACTCCCAT	–	Screening PCR first round
HBVall-R1610a	CTGAGRCCSACTCCCATWGG	–	Screening PCR second round
HBVall-R1610b	GAGAGRCCSACTCCCATWGG	–	Screening PCR second round
Surface/polymerase/X/Core/HDV			
GabonHBV-F	TCTGCGGCGTTTTATCATATACC	+	Real-time PCR-based virus quantification
GabonHBV-P	FAM-TCCTGCTGCTATGCCTCATCTTCTTGTTG-BHQ1	+	
GabonHBV-R	CCCCTCCAGTCCAGGAGAA	–	
HBV-PBP10-rtF	GTGTCTGCGGCGTTTTATCA	+	
HBV-PBP10-rtP	FAM-ACCTCTTGTTCCTGCTGCTAGTCCTCACCT-BHQ1	+	
HBV-PBP10-rtR	CCAGTCCAGGAGAACCAACAA	–	
HDV2s	TCCAGAGGACCCCTTCA	+	
HDV2as	CCGGGATAAGCCTCACT	–	
HDV-Probe	FAM-AGACCGAAGCGAGGAGGAAAGCA-TAMRA	+	
HBV-fw	ACTAGGAGGCTGTAGGCATA	+	
HBV-rev	AGACTCTAAGGCTTCCCG	–	
TBHBV-fw	ATGATTAACAGGGAGGTGG	+	
TBHBV-rev	CCACACATAATCAAGTGCC	–	
RBHBV-fw	CATATGTATTAGGAGGCTGTAGG	+	
RBHBV-rev	CTTCTTCATAGAGAGCTGTGG	–	
HBHBV-fw	CATATGTACTAGGAGGCTGTAGG	+	
HBHBV-rev	TTCCTCATATAAGGCCACC	–	

Sensitivity of the screening assays was determined using 200 μL second World Health Organization international HBV reference standard containing 1 million IU/mL (product code: 97/750; National Institute for Biological Standards and Control) extracted with the MinElute Spin Kit (Qiagen) and an elution volume of 100 μL. The sensitivity of the PCR assay using HBV-F248 at a 95% probability of detection was 41.3 IU/mL blood (95% confidence interval = 29.7–75.9). The sensitivity of the PCR assay using HBVall-F1364 at a 95% probability of detection was 64.7 IU/mL (95% confidence interval = 47.3–112.9). B, C/G/T; BHQ, black hole quencher; FAM, 6-carboxy-fluorescein; M, A/C; R, G/A; S, G/C; W, A/T; Y, C/T.

*First-round 25-μL PCR reactions used the Platinum Taq Kit (Invitrogen) with 5 μL DNA, 400 nM each first-round primers or an equimolar mix of primers of the same polarity, 1 μg BSA, 0.2 mM each dNTP, and 2.5 mM $MgCl_2$. Second-round 50-μL P. Taq reactions were carried out as above and used 1 μL first-round PCR product. PCR reactions were carried out using a touchdown protocol at 95 °C for 3 min, 10 cycles of 15 s at 94 °C and 20 s at 64 °C with a decrease of 1 °C per cycle, and extension at 72 °C for 45 s followed by another 40 cycles at 54 °C annealing temperature.

[†]Numbered after HBV genotype E (GenBank accession no. X75664).

[‡]Numbered after the polymerase of ape HBV (GenBank accession no. NC_001344).

Table S3. Full genome nucleotide distances between the unique bat and prototype hepadnaviruses

		[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]
HBV (New World genotypes)	[1]	8.4								
HBV (Old World/global genotypes)	[2]	13.3–14.7	7.4–12.6							
WMHBV (New World nonhuman primate)	[3]	22.7–23	21.7–22.6	0						
Ape HBV (Old World nonhuman primate HBV)	[4]	12.9–13.5	9.3–12.4	21.2–22.2	5.9–9.8					
TBHBV (New World bat hepadnavirus)	[5]	38.1–38.8	38.8–39.5	39–39.3	38.5–39.5	0.1–2.3				
RBHBV (Old World bat hepadnavirus)	[6]	34.9–35.3	34.9–36	35.7–35.8	35–35.4	40.1–40.4	0.4–0.5			
HBHBV (Old World bat hepadnavirus)	[7]	35.1–35.7	34.3–35.6	35.4–35.4	34.9–35.6	39.6–39.8	18.9–19.1	0		
GSHV, ASHV, WHV (New World rodent hepadnaviruses)	[8]	36.5–37.8	36.3–38.1	36.6–37.8	36.1–37.5	40.3–41.3	36.3–37.2	29.7–30	14.8–16	
SGHBV (Avihepadnavirus)	[9]	55.9–56.4	55.9–56.9	57.1–57.1	55.8–56.2	58.5–58.8	58.4–58.5	57.9–57.9	57.1–57.2	0

The Old World bat hepadnaviruses RBHBV and HBHBV were most closely related, with 19% genomic nucleotide sequence distance. This Old World bat hepadnavirus clade was equidistant from the New World rodent virus clade containing WHV, GSHV, and ASHV and the primate HBV clade, with 35% and 36% nucleotide exchanges, respectively. The sequence distance between bat hepadnaviruses of the Old World and New World was 39%. The genomic distance of the New World bat hepadnavirus TBHBV to primate HBV was 37–39%, and its distance to the New World rodent viruses was 40%. For comparison, the distance between the rodent hepadnaviruses and the primate HBV clade was 36–38%. ASHV, Arctic Squirrel Hepatitis Virus; GSHV, Ground Squirrel Hepatitis Virus.

Table S4. Maximum/minimum amino acid distances between ORFs of the unique bat hepadnaviruses and prototype hepadnaviruses

	Maximum amino acid distance									Minimum amino acid distance								
	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]
Core																		
[1]	0.9									0.9								
[2]	10.8	11.8								4.7	2.4							
[3]	11.4	13.9	0							10.9	9.9	0						
[4]	6.6	10.3	10	4.2						4.2	4.2	8.5	0.9					
[5]	42.7	44.3	44.6	44.9	0					42.7	42.9	44.6	44.3	0				
[6]	29.9	33.3	31.9	30.8	43	0				28.9	28	31.9	29.4	43	0			
[7]	32.7	35.4	34.3	33.2	40.9	15.7	0			31.8	30.3	34.3	32.2	40.9	15.7	0		
[8]	30.8	34.5	31.8	31.3	44	27.8	21.7	9.2		28.9	28	31	28.4	42.4	24.7	21.2	7.8	
[9]	68.6	70.2	68.5	69.8	70.2	70.1	70.7	71.7	0	68	68	68.5	68	70.2	70.1	70.7	69.9	0
LHBs																		
[1]	4.2									4.2								
[2]	19	17								16.7	6.7							
[3]	27.2	27.2	0							26.5	25.2	0						
[4]	19	16.2	25.7	12.6						15.7	9.8	24.2	5.1					
[5]	44.4	46.8	46.2	45.7	2.4					43.6	44.1	45.9	44.4	0.3				
[6]	45	45	43.9	43.2	50.3	0.4				44.2	42.9	43.9	41.6	50	0			
[7]	44.5	45	44.5	43.7	48.8	27.8	0			43.7	42.9	44.5	42.9	48	27.8	0		
[8]	50.9	53.8	51.6	51.8	55.7	45.4	40.7	17.3		49.9	49.2	50.3	48.7	52.5	44.3	40.7	13.2	
[9]	68.2	68.9	70.1	67.7	72.8	69.2	69.2	72.6	0	67.9	67.2	70.1	67	72.1	68.8	69.2	71.6	0
Pol																		
[1]	8.9									8.9								
[2]	16.8	15.8								15.3	8							
[3]	29.7	29.8	0							29.5	28.2	0						
[4]	16.8	14.4	29.4	12.6						14.8	11.1	28.8	7.1					
[5]	46.1	47.6	48	46.9	3.1					45.5	45.6	47.6	45.4	0.1				
[6]	44.3	45.1	44.1	44.3	46.5	1.1				43.7	42.9	43.9	43.4	45.9	0.6			
[7]	44.6	45.2	44	44.6	46.4	22	0			43.8	43.2	44	44	46.2	21.8	0		
[8]	47.6	49.2	48	48.3	48.7	45.4	36.5	23.4		45.5	45.5	46.4	45.5	48.3	43.9	36	20.4	
[9]	66.8	68.6	67.9	67.5	70.1	69.2	70.3	69.5	0	65.9	66.8	67.9	66.3	69.8	69.1	70.3	69.2	0
X																		
[1]	14.9									14.9								
[2]	24.7	22.7								15.6	7.1							
[3]	33.3	34.7	0							32.7	31.3	0						
[4]	18.2	24	34	15.6						14.3	10.4	30	9.7					
[5]	53.8	57.7	57.6	57.7	4.4					50.8	51.5	56.1	51.5	0				
[6]	48.6	51.4	44.3	51.4	53.8	0.7				48.6	46.4	43.6	49.3	52.3	0			
[7]	55.1	53.6	47.1	52.2	56.1	20.6	0			52.2	49.3	47.1	50	54.5	19.9	0		
[8]	56.6	57.4	57.4	58.8	63.1	51.4	43.3	31.2		52.5	50	50	50.7	54.6	48.6	41.1	27.5	
[9]	81	79.8	77.9	79.8	81.9	82.8	86.2	88.4	0	77.4	76.2	77.9	76.2	80.7	82.8	86.2	83.7	0

Distances are shown in a heat map fashion, with shades of green representing lowest distances and shades of red representing highest distances. Groups 5, 6, and 7 contain bat hepadnaviruses. Comparisons within one group excluded tautological analyses of individual viruses with themselves, unless there was only one virus compared, to avoid empty fields. Old World bat hepadnaviruses were generally more related to the primate HBV clade than TBHBV. Minimal amino acid distances between RBHBV/HBHBV and any primate HBV ranged from 28.0% in the core to 43.6% in the X protein. For TBHBV, this range was from 42.7% in the core to 50.8% in the X protein. In analogy to similar genome lengths, ORFs of the Old World bat hepadnaviruses and specifically, HBHBV were closely related to the rodent hepadnaviruses, with minimal amino acid distances ranging from 21.2% in the core protein to 41.1% in the X protein. GenBank accession numbers of analyzed viruses were [1], New World HBV (Genotypes F and H): X69798 and AY090454; [2], Old World/global HBV (Genotypes A–E, G, I, and J): AM282986, AB126581, AF241409, AB117758, AB486012, AB205192, D23678, and AB056513; [3], New World nonhuman primate (WMHBV): AF046996; [4], Old World nonhuman primate (gorilla, Chimpanzee, orangutan, and gibbon): AF193863, AJ131568, AF305327, and AJ131567; [5], New World bat hepadnavirus TBHBV from *Uroderma bilobatum*; [6], Old World bat hepadnavirus RBHBV from *Hipposideros cf. ruber*; [7], Old World bat hepadnavirus HBHBV from *Rhinolophus alcyone*; [8], New World rodent hepadnaviruses (GSHV, ASHV, and WHV): NC_004107, NC_001484, and U29144; and [9], Duck HBV: NC_001344; in X snow goose HBV: AF111000. Snow goose HBV was selected, because it showed the longest X-like ORF within all avihepadnaviruses and an ATG start codon, which is not present in all homologous sequences of avihepadnaviruses.

Table S5. Association between bat hepadnavirus DNA and antibody detection

Serum identity	Hepadnavirus DNA*	Copies/mL†	IFA‡	IFA end-point titer	IFA cross-reactivity§	IFA anti-LHBs reactivity¶	IFA anti-LHBs cross-reactivity‖
Hipposiderid bat GB/09/							
251	Negative		Positive	1:400	Yes	No	No
281	Negative		Positive	1:800	Yes	No	No
293	Negative		Positive	1:100	No	No	No
341	Negative		Positive	1:200	No	No	No
352	Negative		Positive	1:100	No	No	No
359	Negative		Positive	1:400	Yes	No	No
385	Negative		Positive	1:800	Yes	Yes	Yes
256	Positive	1.54E+05	Positive	1:400	Yes	No	No
262	Positive	5.46E+05	Positive	1:1,600	Yes	No	No
301	Positive	8.24E+08	Negative				
303	Positive	6.45E+05	Negative				
Rhinolophid bat GB/09/							
403	Positive	3.26E+03	Positive	1:1,600	Yes	No	No

*In any PCR screening assay.
†In strain-specific quantitative real-time PCR assays.
‡Immunofluoresence assay (IFA) -detecting antibodies against the full homologous virus in bat sera.
§IFA against the full heterologous virus.
¶IFA against the LHBs of the homologous virus.
‖IFA against the LHBs of the heterologous virus.