Hepatitis delta virus (HDV) is a human hepatitis-causing RNA virus, unrelated to any other taxonomic group of RNA viruses. Its occurrence as a satellite virus of hepatitis B virus (HBV) is a singular case in animal virology for which no consensus evolutionary explanation exists. Here we present a mammalian deltavirus that does not occur in humans, identified in the neotropical rodent species Proechimys semispinosus. The rodent deltavirus is highly distinct, showing a common ancestor with a recently described deltavirus in snakes. Reverse genetics based on a tandem minus-strand complementary DNA genome copy under the control of a cytomegalovirus (CMV) promoter confirms autonomous genome replication in transfected cells, with initiation of replication from the upstream genome copy. In contrast to HDV, a large delta antigen is not expressed and the farnesylation motif critical for HBV interaction is absent from a genome region that might correspond to a hypothetical rodent large delta antigen. Correspondingly, there is no evidence for coinfection with an HBV-related hepadnavirus based on virus detection and serology in any deltavirus-positive animal. No other coexisting viruses were detected by RNA sequencing studies of 120 wild-caught animals that could serve as a potential helper virus. The presence of virus in blood and pronounced detection in reproductive and active males suggests horizontal transmission linked to competitive behavior. Our study establishes a nonhuman, mammalian deltavirus that occurs as a horizontally transmitted infection, is potentially cleared by immune response, is not focused in the liver, and possibly does not require helper virus coinfection.

The genus Deltavirus is an unclassified RNA virus taxon that currently includes only one species, the hepatitis delta virus (HDV). This virus is an important human pathogen and the only satellite virus known in animals. For transmission, HDV must acquire an envelope from a coinfected hepatitis B virus (HBV; family Hepadnaviridae, genus Orthohepadnavirus). Following superinfection of HBV-infected individuals, HDV increases the severity of liver disease (1). Initially detected in 1977 (2), HDV is present in about 10% of chronically HBV-infected patients (3). The circular single-stranded RNA genome is ∼1,700 nt long and is packaged together with the small and large hepatitis delta antigens (S-HDAg and L-HDAg) into an HDV ribonucleoprotein particle that buds with the help of HBV envelope proteins from HDV/HBV-coinfected hepatocytes. During replication, a double rolling-circle scheme produces multimeric antigenic RNAs that are self-cleaved into monomers by a viroid-like ribozyme structure. The ligated circular RNA (cRNA) is further transcribed into multimeric genomic RNAs which also undergo self-cleavage and ligation (4). Remarkably, HDV RNA replication is performed by cellular RNA polymerase II (Pol-II), with S-HDAg being essential in this process, as it coactivates Pol-II by possibly acting as a histone mimic (5). The RNA genome of HDV folds into an unbranched rod-like structure due to its high degree of self-complementarity (6). Budding and interaction with HBV envelope proteins require expression of L-HDAg, which is identical to S-HDAg except for a 19-amino acid extension at its C terminus, directed by cellular adenosine deaminase activity that edits the DNA genome copy under the control of a cytomegalovirus (CMV) promoter confirms autonomous genome replication in transfected cells, with initiation of replication from the upstream genome copy. In contrast to HDV, a large delta antigen is not expressed and the farnesylation motif critical for HBV interaction is absent from a genome region that might correspond to a hypothetical rodent large delta antigen. Correspondingly, there is no evidence for coinfection with an HBV-related hepadnavirus based on virus detection and serology in any deltavirus-positive animal. No other coexisting viruses were detected by RNA sequencing studies of 120 wild-caught animals that could serve as a potential helper virus. The presence of virus in blood and pronounced detection in reproductive and active males suggests horizontal transmission linked to competitive behavior. Our study establishes a nonhuman, mammalian deltavirus that occurs as a horizontally transmitted infection, is potentially cleared by immune response, is not focused in the liver, and possibly does not require helper virus coinfection.

The significance of this study is two-fold. Firstly, it highlights the potential for horizontal transmission of deltaviruses, particularly in the neotropical rodent Proechimys semispinosus. Secondly, it challenges the current understanding of deltavirus evolution and transmission patterns, as it presents a novel deltavirus that is unrelated to any other taxonomic group of RNA viruses. This discovery has implications for the study of deltavirus evolution and the potential for cross-species transmission, which is a concern in the context of emerging infectious diseases.

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The authors declare no competing interest.

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Supporting Online Material

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amber codon on the antigenic RNA (7). Posttranslational far-
necylation of the C terminus of L-HDAg is essential for successful
interaction of the HDV ribonucleoprotein particle with HBV en-
velope proteins (8). HDV has one serotype and is divided into eight
distinct genotypes which show only a moderately structured geo-
graphic distribution (9, 10). The distribution of HBV and HDV
genotypes is also only slightly correlated (11). HDV can be exper-
imentally transmitted to woodchucks that carry replicating wood-
chuck hepatitis virus, but has not been observed in feral animals (12,
13). HDV remains the only human RNA virus for which there is no
known replicating counterpart in other animals.

Recently, deltavirus-like sequences have been found in tran-
scriptome data derived from pooled oropharyngeal and cloacal
samples of teals and ducks (14), tissues of boas (Boa constrictor) and
a water python (Lialis mackloti) (15), and various vertebrate and
invertebrate transcriptomes (16). The snake- and duck-associated
sequences, although highly distinct from HDV, show predicted simi-
larity in secondary structure to the HDV ribosome element.

Amino acid motifs known to have functional importance for HDV
replication occur in both sequences. However, the sequences de-
scribed by Chang et al. (16) lack the two ribosome structural ele-
ments and some of the functional motifs, as well as experimental
proof of the viral replication mechanism. Also, the natural history of
disease, if transmissible, is not known for these putative viruses.

Here we describe a unique mammalian deltavirus from the
neotropical rodent species Proechimys semispinosus, tentatively
named rodent deltavirus (RDeV). We initially discovered the
viral RNA following undirected next-generation sequencing of
blood samples. Our study of 763 animals provides epidemi-
ological and virological evidence for a transmissible and prevalent
infection with a delta-like agent in a specific host. Surprisingly,
and in striking contrast to human HDV, we neither find a liver-
specific tropism of RDeV nor epidemiological or functional ev-
idence of coinfection with a hepadavirus in the animals studied.

Results

In an ongoing study in the Panama Canal area, focused on the
effects of habitat disturbance on the infection rate with rodent
Hepacivirus (SI Appendix, Fig. S1) (17), we subjected blood samples
of 120 individuals of P. semispinosus to total RNA sequencing (RNA-seq) using the Illumina MiSeq platform. High bit-score
matches to human HDV were obtained after comparing de novo
assembled sequences with a viral reference library (18). Based on
mapping against a reference alignment of all known HDV geno-
types, we compiled HDV-related sequences covering the whole
deltavirus genome from two individual animals. After aligning
these initial genome assemblies with HDV reference genomes, we
designed an RT-PCR assay targeting a fragment in the coding
region of the rodent deltavirus antigen (RDeAg). The assay
was applied to pooled blood samples from 763 P. semispinosus indi-
viduals. Resolution of positive-testing pools resulted in 30 positive
individuals (overall detection rate 3.9%; 95% CI 2.6 to 5.3%). Only
adult animals were found positive. In addition to P. semispinosus,
we examined blood samples from individuals (n = 183) from 11
other rodent and marsupial species. No species other than P.
semispinosus yielded evidence of RDeV presence (SI Appendix,
Table S1).

Because HDV has a circular genome, we used primer pairs
whose 5′ ends are adjacent on reverse-complementary template
strands, with 3′ ends facing in opposite directions (Fig. 1B and SI
Appendix, Table S2). These primers can generate RT-PCR ampicons covering the near-full deltavirus genome if the template
RNA molecule is circular. This was successful in three samples with
high viral load (Fig. 1C). Genome circularity was further confirmed
by mapping RNA-seq reads from the respective samples to the
initial ampicon sequences, resulting in assemblies with protruding
ends that are identical, matching both ends of the ampicon, and
therefore derived from circular templates. Ten full circular RDeV
genomes and 10 partial genome assemblies were subsequently
obtained from the 30 initially RNA-positive samples by mapping
reads from individual RNA-seq datasets. PCR products for the
other 10 samples produced small fragments of less than 200 bases.
The nucleotide sequences of all 10 full genomes are 97.5 to 99.6% identical. The sequences are available in GenBank under accession
numbers MK598003 to MK598012.

Major structural and functional HDV domains are present in
all recovered RDeV sequences (Fig. 1A and SI Appendix, Fig. S2
and Table S10), including a single open reading frame (ORF) rep-
resenting the delta antigen. A 19-amino acid tail that differen-
tiates the S-HDAg from the L-HDAg in human HDV is present in RDeV, though with a different amino acid composition.
This tail is the result of antigenomic editing at a site corre-
ponding to the amber stop codon in the S-HDAg ORF of human
HDV (SI Appendix, Fig. S3). There is no experimental evidence of RNA editing or large-antigen expression in RDeV,
as discussed below. In addition, the farnesylation motif CXXQ in
the HDV L-HDAg C terminus, required to acquire the HBV-
derived envelope (8), is absent in all P. semispinosus deltavirus
sequences.

Predicted secondary structures of genomic and antigenic ribosome
targets are highly similar between human and rodent delta-
virus (SI Appendix, Fig. S4). The ribosome active site is identical
between rodent and human virus on both antigenomic and ge-
nomic strands. The nuclear export sequence motif and the nuclear
localization signal of S-HDAg are also present in the S-RDeAg.
Evidence for the existence and functionality of the nuclear local-
ization signal is provided by the occurrence of the small delta
antigen in the cell nuclei of HuH7 cells overexpressing S-RDeAg
(Fig. 2A).

Deltavirus Phylogeny. Nucleotide and amino acid sequences were
compared as summarized in SI Appendix, Fig. S2 and Table S3.
Phylogenies were calculated based on full-genome nucleotide
sequence alignments, as well as translated small antigen-
coding sequences. Both tree topologies are equivalent and the
distinction of RDeV from human HDV is clear (the full-genome
phylogeny is shown in Fig. 3). The nonhuman deltaviruses are
highly diverged and therefore chosen to root the phylogeny. The
snake deltavirus (SDeV) clusters with the rodent deltavirus, and
both structural and functional well-supported sister group to
human HDV, with HDV genotype 3 being the most divergent
among HDV genotypes. All other nonhuman deltaviruses group
together consistently, although without significant bootstrap
support among subclades, owing to their high sequence diver-
gence. RDeV is most closely related to human HDV, as aver-
aged over all 1,000 bootstrap replicate trees (Fig. 3).

Organ Distribution of RDeV RNA and Protein Detection. To obtain
insight into the infection pattern caused by the rodent deltavirus,
we tested for viral RNA in organs of 18 animals that were found
dead during sampling (note that we followed a noninvasive
sampling approach and animals could not be purposefully eutha-
nized during our study). One of these animals tested RDeV RNA-
positive in all available organs (liver, kidney, lung, heart, and
small intestine), but its organ-specific RNA concentrations
did not suggest specific virus replication in the liver (SI Appendix,
Table S4). Also, a commercial HDV antigen enzyme-linked
immunosorbent assay that we found to cross-detect with RDeV
did not identify organ-specific protein expression in the liver,
lung, small intestine, heart, or kidney of this animal (SI Appendix,
Fig. S5A). To further understand potential virus excretion, fecal
samples from 822 individuals were tested by RT-PCR, 10 of
which were positive for RDeV RNA (the numbers of individuals
tested for each type of material are summarized in SI Appendix,
Table S5). This 1.2% detection rate was significantly lower than
that in blood samples (χ² test, P < 10⁻³). The average virus
concentration in fecal samples was $2.9 \times 10^9$ RNA copies per gram, with a maximum concentration of $2.2 \times 10^{10}$ copies per gram. Average and maximum concentrations in blood were $1.8 \times 10^8$ and $2.1 \times 10^9$ copies per milliliter.

**RDeV Genome Replication.** Rodent deltavirus genome replication was confirmed by Northern blot analysis, as well as by clonal expansion of RDeV-transfected cells. The full RDeV genome was cloned as a tandem head-to-tail fusion dimeric construct in genomic (minus-strand) orientation downstream of a cytomegalovirus (CMV) promoter (see the construct map in SI Appendix, Fig. S6) and transfected into HuH7 cells. Antigenomic RDeV RNA as a marker of ongoing RDeV replication was detected in transfected cells at all time points (Fig. 2F). The expression of antigenomic RDeV RNA was accompanied by a robust synthesis of genomic RDeV RNA (SI Appendix, Fig. S5D). Massive clonal expansion of RDeV-transfected cells verifies the autonomous replicating nature of RDeV, even exceeding HDV clonal expansion as determined by immunofluorescence staining (Fig. 2G).

**Deltavirus Antigens and Editing of the Amber Codon.** One key feature of HDV is the expression of two viral proteins (S-HDAg and L-HDAg) from a single ORF. A 19-amino acid carboxyl-terminal extension differentiates the S-HDAg (195 codons) from the L-HDAg (214 codons) in human HDV. This extension is the result of antigenomic editing by cellular adenosine deaminase acting on RNA 1 (ADAR1) at a site corresponding to the amber stop codon in the S-HDAg ORF of the circular antigenomic HDV RNA, edited from a UAG to a UIG codon. Following HDV RNA replication, the UIG codon is converted into a tryptophan codon (UGG) (SI Appendix, Fig. S3) during subsequent HDV messenger RNA (mRNA) synthesis, allowing expression of the L-HDAg (20). Interestingly, a 19-amino acid carboxyl-terminal extension is present in RDeV, though different in 15 of the 19 amino acids. To investigate potential RDeV large antigen (L-RDeAg) expression, the full RDeV genome was cloned as a tandem head-to-tail fusion construct in genomic (minus-strand) orientation downstream of a CMV promoter (see the construct map in SI Appendix, Fig. S6) and transfected into HuH7 cells. As positive controls, S- or (hypothetical) L-RDeAg was expressed under the control of a CMV promoter. Specific S- or L-RDeAg expression was examined with antibodies from rabbits immunized with synthetic peptides against S-RDeAg (Fig. 2A) or the putative 19-amino acid extension of L-RDeAg (Fig. 2B). The tandem genome construct expressed S-RDeAg but not L-RDeAg (Fig. 2A and B).

Corresponding results were obtained by Western blot analysis of RDeV dimer transfection experiments using a broadly cross-reactive human anti-HDV serum (Fig. 2H, Upper). Evidence for the absence of RNA editing on the RDeAg ORF was also obtained from RNA-seq data. Mapping of reads from total RNA blood extracts of infected animals and from transfected RDeV dimeric genome constructs in cell culture yielded only unedited reads, whereas both edited and unedited reads were detected for human HDV in a control experiment (SI Appendix, Fig. S3). In summary, there is no experimental evidence of
Search for Signs of Hepadnaviral Coinfection. Because human HDV transmission requires an envelope protein provided by an active coinfection with HBV, we subjected all 763 *P. semispinosus* blood samples to PCR screening for hepadnaviruses. The assay was designed to detect all mammalian orthohepadnaviruses, including those from rodents, bats, and primates (21). None of the samples tested positive. Specific reanalysis of all available transcriptome datasets from the present study did not yield any evidence for orthohepadnaviral genomes in a basic local alignment search tool (BLAST) analysis. All blood samples with sufficient volume, whether RNA-positive or -negative for RDeV (*n* = 68), were tested for antibodies against woodchuck HBV core antigen (WHcAg). This antigen was chosen because the woodchuck is most closely related to *Proechimys* among known HBV hosts. In previous studies, we demonstrated that anti-HBc antibodies broadly cross-react among HBVs from different hosts, even across mammalian orders (21); so it is likely that WHcAg would be bound by antibodies against a possible HBV in *Proechimys*. However, no such antibodies were found in any of the tested samples (Fig. 2D).

Investigation of Hepivirus as Potential Cofactor. Experimental evidence shows that apart from HBV, other viruses, including hepatitis C virus (genus *Hepacivirus*), can provide envelope proteins for human HDV (22). As we previously detected a high prevalence of hepacivirus in the rodents studied (17), this possibility was investigated. Overall, we found 4 out of 30 RDeV RNA-positive rodents in which hepacivirus was not detected by the tests initially applied (SI Appendix, Table S6). To exclude having missed hepacivirus detection, we performed RT-PCR assays specifically designed for the E1, NS3, and NS5B genes of *P. semispinosus* hepacivirus to those samples. All four animals were confirmed to be hepacivirus RNA-negative by these assays as well as by RNA-seq read mapping against *P. semispinosus* hepacivirus. Statistical analysis of the degree of dependency between deltavirus and hepacivirus infection in individuals was conducted, providing no support for a dependency between the two infections (SI Appendix, Table S6; *χ*² test, *P* = 0.24). Also, analyses of sampling site-specific deltavirus and hepacivirus detection rates (SI Appendix, Table S7) did not reveal any correlation between deltavirus and hepacivirus (Pearson’s correlation coefficient, *p* = 0.15), arguing against a linear correlation. Logarithmic and linear regression fits did not yield significant associations either, suggesting that the two viruses follow different patterns of distribution. We conclude that

Total RNA was isolated 4, 6, 8, and 10 d posttransfection (dpt) and subjected to a 1% denaturing formaldehyde agarose gel. After blotting, the RNA was hybridized with a single-stranded oligonucleotide probe labeled with [*γ-32P*] ATP and T4 polynucleotide kinase at the 5′ end. In vitro-transcripted antigenomic RDeV RNA was used as positive control. (G) Immunofluorescence staining of HuH7 cells transfected with expression plasmids containing an intact dimer of the RDeV genome.
L-RDeAg was cloned and transiently expressed in Vero B4 cells, males tested positive for anti-RDeAg antibodies (RDeV causes an immune reaction in 1:1,000 was 8,500 RNA copies per microliter of blood (those samples that tested antibody-positive up to a dilution of 1:1,000 was 1:1,000 was 8,500 RNA copies per microliter of blood. The average RDeV RNA concentration in only antibodies (Fig. 2 against expressed S-RDeAg confirmed the specificity of serum dilution, testing positive in 5 cases. Western blots of these sera S8). The 20 positive sera were additionally tested at 1:1,000 di-

replication. Elimination is also suggested by the occurrence of adaptive immune response can potentially limit or eliminate viral transmission. The observed predominant infection of adult males (24) and thus (relatively) competitively benefit from an-

environmental changes (24) and thus (relatively) competitively benefit from anthropogenic habitat disturbance. We have previously noted a positive correlation between site-specific P. semispinosus population density and the detection rate of hepacivirus infection (SI Appendix, Table S7) (17). For rodent deltavirus, a different site-specific distribution was observed (SI Appendix, Table S7). Highest detection rates were identified in continuous forest sites that have largely preserved the primordial habitat composition that existed before the flooding of the Gatún Lake area during the construction of the Panama Canal. Lower detection rates were found on forested islands, and minimal rates in forest fragments surrounded by agriculture. To identify

environmental factors that might correlate with rodent deltavirus transmission, host- and habitat-specific properties were examined under two different logistic regression models (SI Appendix, Table S9). RDeV infection was found to be positively correlated with male sex and reproductive activity, as well as with inhabiting continuous forest sites with undisturbed habitat. In addition to the variables shown in SI Appendix, Table S9, other habitat-specific factors such as population density, canopy height and coverage, and understory density did not add explanatory power to the analysis. The inclusion of site-specific data on mosquito diversity narrowed the sample size to n = 686 individuals for sampling sites where these data were available. There was no detectable correlation between mosquito diversity and RDeV detection rate.

Discussion

The present results enhance our understanding of the evolution of hepatitis delta virus, a satellite virus to HBV and until recently the only human RNA virus with no known counterpart in other animals. The first question that must follow the discovery of novel deltavirus-like sequences in rodent, reptile (15), and putatively avian (14) and other vertebrate and invertebrate hosts (16) is whether these represent transmissible viruses as opposed to, for example, endogenous viral elements. Hetzel et al. (15) demonstrated protein expression in organs of those snakes in which viral sequences were detected, and Szirovicsza et al. (25) showed that snake deltavirus can replicate in several cell lines. Our reverse genetic and clonal expansion experiments now prove the existence of mammalian nonhuman deltavirus genomes as self-replicating viral entities, in which genomic replication involves an internal initiation of replication. Moreover, correlations of serology and virus detection in a natural animal population of considerable size demonstrate that rodent deltavirus infection is acquired during the course of life and is likely to be cleared by an active immune response. Previous observations on the snake deltavirus corroborate this infection pattern, showing that although snake deltavirus infection was found in both maternal and offspring boa (15), not all offspring were infected, and snakes with no kinship to the mating pairs exhibited both the presence and absence of the virus. Horizontal transmission seems likely in both rodent and reptile deltavirus. The significantly lower detection rate of viral RNA in stool samples compared with blood suggest blood-borne rather than fecal-oral transmission. The observed predominant infection of adult males is compatible with transmission through competitive behavior.

The novel deltaviruses associated with diverse hosts warrant considerations regarding evolutionary origins. Phylogeny suggests that fish- and amphibian-associated viruses are most distant from other known deltaviruses, while snake and rodent viruses are significantly closer to the HDV common ancestor. Considering the relationship of these hosts, the tree topology seems to disfavor three anti-RDeAg antibody-positive but RDeV RNA-negative animals.

Ecological Factors That Influence Rodent Deltavirus Infection. The study area in the Barro Colorado Nature Monument consists of islands and peninsulas with different densities of populations of P. semispinosus, a generalist species that can adapt to environmental changes (24) and thus (relatively) competitively benefit from anthropogenic habitat disturbance. We have previously noted a positive correlation between site-specific P. semispinosus population density and the detection rate of hepacivirus infection (SI Appendix, Table S7) (17). For rodent deltavirus, a different site-specific distribution was observed (SI Appendix, Table S7). Highest detection rates were identified in continuous forest sites that have largely preserved the primordial habitat composition that existed before the flooding of the Gatún Lake area during the construction of the Panama Canal. Lower detection rates were found on forested islands, and minimal rates in forest fragments surrounded by agriculture. To identify environmental factors that might correlate with rodent deltavirus transmission, host- and habitat-specific properties were examined under two different logistic regression models (SI Appendix, Table S9). RDeV infection was found to be positively correlated with male sex and reproductive activity, as well as with inhabiting continuous forest sites with undisturbed habitat. In addition to the variables shown in SI Appendix, Table S9, other habitat-specific factors such as population density, canopy height and coverage, and understory density did not add explanatory power to the analysis. The inclusion of site-specific data on mosquito diversity narrowed the sample size to n = 686 individuals for sampling sites where these data were available. There was no detectable correlation between mosquito diversity and RDeV detection rate.

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Immune Reaction in RDev-Infected Animals. To determine whether RDeV causes an immune reaction in P. semispinosus, serum samples were subjected to an indirect immunofluorescence assay. L-RDeAg was cloned and transiently expressed in Vero B4 cells, as described (23). Fig. 2C shows the reactivity of antibodies from human sera against L-RDeAg, along with examples of reactive and nonreactive P. semispinosus sera. At a dilution of 1:100, 17 of 30 RDeV RNA-positive and 3 of 115 RDeV RNA-negative animals tested positive for anti-RDeAg antibodies (P < 10−6; χ2 test). To exclude RNA degradation in the case of antibody-positive samples for which RDeV RNA was not detected, we applied a real-time RT-PCR assay to detect RNA transcripts of a host housekeeping gene (TATA-binding protein) (SI Appendix, Table S8). The 20 positive sera were additionally tested at 1:1,000 dilution, testing positive in 5 cases. Western blots of these sera against expressed S-RDeAg confirmed the specificity of serum antibodies (Fig. 2E). The average RDeV RNA concentration in all anti-RDeAg-positive sera was 262,400 RNA copies per microliter of blood. The average RDeV RNA concentration in only those samples that tested antibody-positive up to a dilution of 1:1,000 was 8,500 RNA copies per microliter of blood (SI Appendix, Fig. S5C). These concentrations were significantly different (P < 0.05, Mann-Whitney U test), which suggests that an adaptive immune response can potentially limit or eliminate viral replication. Elimination is also suggested by the occurrence of
virus-host codivergence. Besides, the duck-associated virus was not detected in an internal organ but in cloacal and oropharyngeal swabs, leaving open the possibility of contamination from alimentary sources (14). The phylogenetic branch pattern supports previous work on human HDV phylogeny, suggesting that HDV genotype 3 stems from the oldest common ancestor of human HDV genotypes (26). As this ancestor is more closely related to the rodent virus than to any of the nonmammalian deltaviruses, this would be compatible with the hypothesis that human and rodent deltaviruses have codiverged with mammalian lineages. Deltaviruses in other mammals may remain to be discovered, but equally may now be extinct. A hypothesis of codivergence would be corroborated by the discovery of at least one more mammalian virus whose phylogenetic position reflects that of its host in relation to humans and rodents. The clustering of rodent and snake viruses might then be interpreted as a sign of cross-species acquisition from mammals. It is noteworthy that the parental host carrying the snake deltavirus stemmed from Panama, like the rodents studied here (15). Human HDV genotype 3 is also found in South and Middle America, but this alone should not be taken as support to claim cross-host transmission.

The dependence of HDV on HBV coinfection for transmission may have evolved only in a mammalian viral lineage leading to human HDV, given that hepadnaviruses occur in diverse mammalian species, including rodents, bats, and particularly New World primates (21, 27, 28). Based on the lack of large-antigen expression and a farnesylation motif, both necessary for interaction with hepadnaviral envelope proteins, as well as on evidence from epidemiological and serological data, we can exclude the presence of HBV coinfection in deltavirus-infected P. semispinosus. Neither HBV DNA nor anti-HBc antibodies were detected in any animals infected with the deltavirus. In all other deltavirus studies, no sequences of coinfecting hepadnaviruses were found in transcriptome data. Other coinfecting viruses were identified in each of these studies, designating their envelope proteins as a potential source for an RDeV envelope (14–16). As very recently shown by Szövéczta et al. (25), snake deltavirus can produce infectious viral particles in vitro by utilization of envelope proteins from arenaviruses and orthohantaviruses.

In the present study, we investigated the potential influence of hepacivirus infection. Despite a high hepacivirus prevalence in the studied rodents (ca. 80% (17)), we found four deltavirus RNA-positive individuals lacking a hepacivirus coinfection. Human HDV has been found to produce infectious particles in vitro with the help of an envelope protein from hepatitis C virus, with the required presence of an L-HDAg farnesylation motif (22). Given the absence of this motif in the rodent delta antigen and the lack of a hepacivirus coinfection in some individuals carrying deltavirus RNA, we do not consider deltavirus infection to depend on hepacivirus infection in these rodents. In addition, we screened individual RNA-seq datasets from 120 animals and did not identify any other infectious viral agent in any deltavirus RNA-positive P. semispinosus. There are multiple alternatives that may have resulted in rodent deltavirus transmission. One possibility is the presence of a currently unknown virus, bacterium, or integrated sequence that provides an envelope protein to rodent deltavirus. Furthermore, recent advances in cellular cross-communication have revealed the exchange of extracellular vesicles containing mRNA, circRNA, and other noncoding RNA molecules (29). It should be investigated whether rodent deltavirus might employ such pathways for transmission.

In their study of snake organ samples, Hetzel et al. (15) found two bands of ca. 20 and 27 kDa in Western blots using an anti-S-SDeAg serum. While the larger band might correspond to a larger protein variant, expression of an actual L-SDeAg was not confirmed by specific anti-L-SDeAg immunoblots, analyses of RNA editing, or experimental studies of gene expression. The present data, based on all these approaches, show that the related rodent virus does not express a large delta antigen, suggesting that the use of L-HDAg may have evolved only in connection with the utilization of the HBV envelope. In this scenario, the encounter with hepadnaviruses may have selected for viral variants with a farnesylation signal in an L-HDAg tail that is expressed by an initially low-level editing activity or stop codon readthrough, allowing these viruses to exploit a novel transmission opportunity by acquisition of an HBV envelope. The sequence information from the present study can now be used in directed searches for deltaviruses in other mammalian species, even in the absence of HBV infection.

Alternative hypotheses of HDV origin have suggested either a viroid origin (perhaps via an insect vector) or derivation from a host DIPA gene (reviewed in ref. 11). A viroid origin is supported by the structural similarity of the HDV ribozyme to plant viroid ribozymes, the genome circularity of both HDV and viroids, and the interaction with common cellular proteins (30). Derivation from the human genome has been suggested based on the incomplete set of genes essential for replication, the occurrence of HDV-like ribozyme elements in the human genome (31), and the interaction of HDV with a human-derived protein (DIPA) (32). The deltavirus findings do not increase or decrease support for the viroid origin hypothesis but force us to consider the DIPA hypothesis from a wider perspective. The rodent deltavirus ORF shows 30% nucleotide and 22% amino acid identity when compared with the DIPA ORF in mice and rats, at which levels similarity due to chance must be considered as a likely explanation (33). In addition to these caveats, the present results suggest that other mammalian deltaviruses existed before the origin of human HDV, and that precursors to these mammalian viruses existed in other vertebrates (14–16). Alternate scenarios, specifically the derivation of nonhuman deltaviruses from human HDV precursors, seem unlikely, considering the genetic distances between these viruses, the relatively short time span available for the evolution of such diversity in the case of a human origin, and the requirement that the human virus infect several nonhuman hosts. It appears instead that deltavirus originated as an exogenous infectious agent that established acute infection in mammals. The present study provides a basis for the evolution of HDV, confirming the general understanding that RNA virus diversity evolved along the major evolutionary lineages of their hosts (34).

**Methods**

Animals were captured using Sherman traps in the Barro Colorado Nature Monument, Panama (17). RNA-seq experiments were done on an Illumina MiSeq instrument. RT-PCR followed standard protocols, including for circular genome amplification. Immunoﬂuorescence assay (IFA) serology was based on cells overexpressing the L-RDeV or woodchuck WHC antigen under the control of a CMV promoter. Viral full RDeV genome complementary DNA was cloned as a monomer or head-to-tail dimer under the control of a CMV promoter. A full description of methods is provided in SI Appendix, Materials and Methods.

All fieldwork was carried out with full ethical approval (Smithsonian Institutional Animal Care and Use Committee protocols 2013-0401-2016-A1-A7 and 2016-0627-2019-A2) and samples were exported to Germany with permission from the Panamanian government (SEX/A-21-14, SE/A-69-14, SEX/A-22-15, SEX/A-24-17, SEX/A-120-16, and SEX/A-52-17).

**Data Availability.** The rodent deltavirus complete genome sequences described here are available in GenBank under accession numbers MK598003 to MK598012.

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