Short '1.2× genome' infectious clone initiates deltavirus replication in *Boa constrictor* cells

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1 ABSTRACT

2 Human hepatitis D virus (HDV), discovered in 1977, represented the sole known deltavirus for
3 decades. The dependence on hepatitis B virus (HBV) co-infection and its glycoproteins for infectious
4 particle formation led to the assumption that deltaviruses are human-only pathogens. However, since
5 2018, several reports have described identification of HDV-like agents from various hosts but without
6 co-infecting hepadnaviruses. Indeed, we demonstrated that Swiss snake colony virus 1 (SwSCV-1)
7 uses arenaviruses as the helper for infectious particle formation, thus shaking the dogmatic alliance
8 with hepadnaviruses for completing deltavirus life cycle. *In vitro* systems enabling helper virus-
9 independent replication are key for studying the newly discovered deltaviruses. Others and we have
10 successfully used constructs containing multimers of the deltavirus genome for the replication of
11 various deltaviruses via transfection in cell culture. Here, we report the establishment of deltavirus
12 infectious clones with 1.2× genome inserts bearing two copies of the genomic and antigenomic
13 ribozymes. We used SwSCV-1 as the model to compare the ability of the previously reported “2×
14 genome” and the “1.2× genome” plasmid constructs/infectious clones to initiate replication in cell
15 culture. Using immunofluorescence, qRT-PCR, immuno- and northern blotting, we found the 2× and
16 1.2× genome clones to similarly initiate deltavirus replication *in vitro* and both induced a persistent
17 infection of snake cells. We hypothesize that duplicating the ribozymes facilitates the cleavage of
18 genome multimers into unit-length pieces during the initial round of replication. The 1.2× genome
19 constructs enable easier introduction of modifications required for studying deltavirus replication and
20 cellular interactions.

21 **IMPORTANCE**

22 Hepatitis D virus (HDV) is a satellite virus infecting humans with strict association to hepatitis
 23 B virus (HBV) co-infection because HBV glycoproteins can mediate infectious HDV particle
 24 formation. For decades, HDV was the sole representative of deltaviruses, which had led to hypotheses
 25 suggesting that it evolved in humans, the only known natural host. Recent sequencing studies have
 26 led to the discovery of HDV-like sequences across a wide range of species, representing a paradigm
 27 shift in deltavirus evolution. Molecular biology tools such as infectious clones, which enable initiation
 28 of deltavirus infection without helper virus, are key to demonstrate that the recently found deltaviruses
 29 are capable of independent replication. Such tools will enable identification of the potential helper
 30 viruses. Here, we report a 1.2× genome copy strategy for designing plasmid-based infectious clones
 31 to study deltaviruses and to demonstrate that plasmid delivery into cultured snake cells sufficiently
 32 initiates replication of different deltaviruses.

INTRODUCTION

Hepatitis D virus (HDV) is a unique human pathogen. Three years after its discovery in 1977 in liver specimens of chronically hepatitis B (HBV) infected patients (1), Rizzetto and colleagues identified it as a satellite virus of HBV (2). One can contract HDV in two different ways, either through acute co-infection with HBV or through superinfection as a chronic HBV carrier. HBV and HDV co-infection is clinically more severe than HBV mono-infection; however, the infection usually resolves resulting in the clearance of both viruses. Superinfection of a chronic HBV carrier by HDV results in the most severe form of viral hepatitis; these patients often face hepatic cirrhosis and development of hepatocellular carcinoma (3). HDV is a satellite virus that utilizes the envelope proteins of HBV to assemble infectious viral particles; however, the replication of HDV within the host cell proceeds independently of HBV (4). The single-stranded RNA genome of HDV is around 1.7 kilonucleotides long although because of high self-complementary, it forms a double-stranded rod-like structure (5, 6). Within the cell, HDV gives rise to three different RNA species: the genome, the antigenome, and the mRNA. The antigenome is the exact complement of the genome, while the mRNA mediates the expression of the delta antigen (DAg), the sole protein encoded by the HDV genome (5, 7). During the viral life cycle, the DAg is present in two different forms, small (SDAg) and large DAg (LDAg) (8). Cellular editing mediated by adenine deaminase 1 (ADAR1) converts the amber stop codon of the SDAg on the antigenomic strand to a tryptophan codon, thus allowing the extension of the protein by 19 additional amino acids (9, 10). The two forms of the protein not only differ in their length but they also have vastly different roles. The SDAg promotes viral replication, while the LDAg inhibits it and shifts the viral life cycle towards packaging (8). The host cell's RNA polymerases mediate the replication of the HDV genome, which occurs via a double rolling circle mechanism (11). A curious feature of HDV is the presence of ribozyme sequences in both its genome and antigenome (12). The ribozymes cut the multimeric HDV RNA species produced during the rolling circle replication into unit-length pieces (11).

HDV was the sole representative of the unassigned genus *Deltavirus* until 2018 (13). The discovery of HDV-like sequences in birds and snakes in 2018 marked the beginning of a new chapter in deltavirus research by broadening the potential host spectrum (14, 15). First, an HDV-like sequence was discovered in waterfowl during a meta-transcriptomic study without traces of HBV or hepadnaviral reads but influenza A virus reads instead (14). Co-incidentally, we reported the identification of a deltavirus in *Boa constrictors* (Swiss snake colony virus 1, SwSCV-1, initially known as snake deltavirus, SDeV) in co-infection with reptarena- and hartmaniviruses but in the absence of hepadnaviral reads (15). The next paradigm shift of deltavirus research was the broadening of the scope of putative HDV helper viruses to include vesiculo-, flavi- and hepaciviruses (16). The researchers further showed that HDV forms infectious particles using the glycoproteins (GPs) of the aforementioned viruses, not only in liver, but also in kidney cells. Soon after, we managed to isolate SwSCV-1 along with co-infecting reptarena- and hartmaniviruses from brain homogenates of an infected snake (17). We utilized persistently SwSCV-1 infected cell cultures to demonstrate that reptarena- or hartmanivirus superinfection results in the egress of infectious SwSCV-1 particles, adding to the evidence that reptarena- and hartmaniviruses are the likely helpers of the virus (17). We further demonstrated that expression of arena- and orthohantavirus GPs in the persistently infected cultures also induces infectious particle formation (17). The aforementioned reports provoked several meta-transcriptomic studies, resulting initially in the identification of deltaviruses in subterranean termites, fish, Asiatic toad, and Chinese fire belly newt in 2019 (18). In 2020, Paraskevopoulou and colleagues found a deltavirus in Tome's spiny rats without sequences for a helper virus, but including evidence for autonomous replication in cell culture and the host (19). Metatranscriptomic studies revealed more deltaviruses in common vampire bats, a lesser dog-like bat, white-tailed deer, eastern woodchuck, passerine birds, zebra finch, lantern fish, and amboli leaping frogs (20-22). The recent findings have sparked deltavirus research and raised questions about the evolutionary origin of

deltaviruses (23-25). The identification of deltaviruses across several taxa led to the establishment of a new realm, *Ribozyviria*, with family *Kolmioviridae* including eight genera (26, 27).

A number of tools to initiate HDV replication *in vitro* and to mimic infection have been developed over the years. The first and probably most widely used method is the transfection of cells with a plasmid containing a trimer of the entire HDV genome under the control of the simian virus 40 promoter (4). In theory, the transfection results in multimeric RNA transcripts of the HDV genome, which resembles the rolling circle replication of the virus during actual infection. It is also possible to initiate the replication by transfecting a vector carrying a monomer of the HDV genome, but in this case, an HDAg expressing plasmid needs to be provided *in trans* (28). Macnaughton and Lai showed that direct RNA transfection with HDAg provided *in trans* can overcome the use of artificial DNA intermediates to initiate replication. Interestingly, they found that transfection with 1.2× genome-length RNA resulted in the most efficient replication, perhaps because the ribozymes at both ends of the genome were in duplicate (29). In our previous study, we constructed a plasmid containing the entire SwSCV-1 genome in duplicate in head-to-tail fashion (17), following the idea of the trimeric HDV constructs (4). The transfection of the “2×” construct initiates efficient SwSCV-1 replication in snake cells, eventually leading to persistent infection (17). The ‘genome-dimer or 2×’ plasmid principle was also successfully adapted for Tome's spiny rat virus 1 (TSRV-1), *Taeniopygia guttata* deltavirus, and *Marmota monax* deltavirus (19, 21). Here, we describe a novel construct containing 1.2× SwSCV-1 genome, which in a manner similar to the previously described 2× SwSCV-1 infectious clone (17), initiates virus replication, produces infectious particles upon superinfection with Haartman institute snake virus 1 (HISV-1) and results in persistent infection of the cells. We based our 1.2× infectious clone on the RNA transfection studies of Macnaughton and Lai (29) with the aim to generate shorter DNA constructs to facilitate introduction of mutations or modifications to the virus genome. Additionally, we wanted to eliminate the T7 promoter we used in

106 the 2× SwSCV-1 infectious clone to avoid the risk of DAg expression via this promoter due to cellular
107 polymerases, which is reported to occur in mammalian cells (30, 31).

MATERIALS AND METHODS

Cell culture and superinfection

The study made use of previously described cultured *Boa constrictor* kidney cells, I/1Ki, (32) and persistently SwSCV-1 infected I/1Ki cells, I/1Ki-Δ (17). The cells were maintained in Minimal Essential Medium Eagle (Sigma Aldrich) supplemented with 10% fetal bovine serum (Gibco), 200 mM L-glutamine (Sigma Aldrich), 100 μg/ml of streptomycin (Sigma Aldrich), and 100 U/ml of penicillin (Sigma Aldrich) in an incubator at 30°C with 5% CO₂.

To establish another persistently SwSCV-1 infected I/1Ki cell line, designated I/1Ki-1.2×Δ, we transfected I/1Ki cells with a plasmid containing 1.2 copies of the SwSCV-1 genome (described below) and maintained the cells as described above and earlier (17).

To study infectious particle formation of the SwSCV-1 infected cell lines, we conducted superinfection studies with HISV-1 (33), earlier demonstrated to efficiently serve as the helper for SwSCV-1 (17). The superinfection studies and detection followed the protocol described (17).

Plasmids and cloning

We ordered synthetic genes from Gene Universal for the 1.2× copies of the following deltaviruses: SwSCV-1 (initially known as snake deltavirus, GenBank accession number: NC_040729.1)(17), Tome's spiny rat virus 1 (TSRV-1, initially known as rodent deltavirus, MK598005.2)(19), dabbling duck virus 1 (DabDV-1, initially known as avian HDV-like agent, NC_040845.1)(14), Chusan Island toad virus 1 (CITV-1, initially known as toad HDV-like agent, MK962760.1)(18), and HDV-1 (M21012.1). Each synthetic gene, flanked by EcoRV restriction sites, contained the full genome with additional nucleotides to include both the genomic and antigenomic ribozymes twice (Figure 1). Subcloning of the constructs into pCAGGS followed the procedures described in Szirovicza et al, 2020 (17). Briefly, FastDigest EcoRV (ThermoFisher Scientific) served to restriction digest the inserts, followed by purification after agarose gel electrophoresis using the GeneJET Gel extraction

kit (ThermoFisher Scientific). T4 DNA ligase (ThermoFisher Scientific) served to ligate the purified inserts into pCAGGS/MCS plasmid (34) purified from agarose gel by the GeneJET Gel extraction kit (ThermoFisher Scientific) after linearization with Fast Digest EcoRI and XhoI (ThermoFisher Scientific) restriction enzymes and T4 DNA polymerase (ThermoFisher Scientific) blunting. We plated chemically competent *E. coli* (DH5 α strain) transformed with the ligation products on Luria-Broth (LB) agar plates with 100 μ g/ml of ampicillin, and incubated overnight (O/N) at 37°C. We picked single colonies and transferred them into 5 ml of LB medium (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract), followed by O/N incubation at 37°C (220 rpm), after which the GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific) served for plasmid isolation from 2 ml of the O/N culture. The DNA Sequencing and Genomic Laboratory, Institute of Biotechnology, University of Helsinki performed Sanger sequencing of the preparations and confirmed these to contain an insert of the correct size. For each virus, we selected two clones, one each with the insert in genomic and in antigenomic orientation for plasmid stock preparation using ZymoPURE II Plasmid Maxiprep Kit (Zymo Research).

Transfection

Lipofectamine 2000 (ThermoFisher Scientific) reagent served for transfection of I/1Ki cells as described (17, 35). Briefly, we mixed 500 ng of plasmid DNA in 50 μ l of OptiMEM (ThermoFisher Scientific) and 3 μ l of Lipofectamine 2000 in 47 μ l of OptiMEM (ThermoFisher Scientific) by pipetting up and down, and allowed the complexes to form for 15-30 minutes at room temperature (RT). We added 1 ml of trypsinized cells (suspension containing approximately 1.8 $\times 10^6$ of cells per ml) to the mixture and allowed the suspension stand at RT for 15-30 min before plating. At 5-6 h post plating, we replaced the transfection mixture by fully supplemented medium and incubated the cells as described above. We scaled up the above reaction volumes depending on the amount of cells needed for each experiment.

Western blot (WB)

For WB, we washed the cells grown on plates or flasks twice with PBS, scraped them into PBS, pelleted by centrifugation ($500 \times g$, 3-5 min), lysed the cell pellets by RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Tx-100, 0.1% SDS, 0.5% sodium deoxycholate, protease inhibitor cocktail), and measured the protein concentration using the PierceTM BCA Protein Assay Kit (ThermoFisher Scientific). For comparing DAGs of different deltaviruses, we collected from a 12-well plate non-transfected I/1Ki cells and those transfected with FWD and REV constructs at 4 days post transfection in 100 μ l of Laemmli sample buffer after two washes with PBS. We separated an equal amount of protein (or volume, 30 μ l/lane, for the experiments done on 12-well plate) for each sample on SDS-PAGE using 4–20% Mini-PROTEAN[®] TGX gels (Bio-Rad), and immunoblotted as described (36). We used 1:4000 dilution for the rabbit anti-SDAg primary antibody (15) or 1 μ g/ml of IgG affinity purified from anti-SwSCV-1 rabbit antiserum (17) using recombinant human DA_g as described (17), 1:10,000 for AlexaFluor 680 donkey anti-mouse (IgG) (ThermoFisher Scientific), and 1:10,000 for IRDye 800CW donkey anti-rabbit (IgG) (LI-COR Biosciences). The Lab VisionTM pan-actin mouse monoclonal antibody (ThermoFisher Scientific) used at a 1:200 dilution served for detection of β -actin. The Odyssey Infrared Imaging System (LI-COR Biosciences) was employed to record the results.

Immunofluorescence staining

For immunofluorescence (IF) staining, we plated the cells on collagen coated ($10 \mu\text{g}/\text{cm}^2$ type I rat tail collagen [BD Biosciences] in 25 mM acetic acid, O/N at 4°C) on CellCarrier-96 Ultra plates (PerkinElmer). After the removal of culture media, cells were fixed by incubation in 4% paraformaldehyde in PBS for ~15 min at RT. The IF staining followed the protocol described (17). We used directly labelled anti-SDAg-AF488 (17) at 1:500 dilution, rabbit anti-SDAg antiserum at 1:4,000 dilution, and 1:1,000 of either Alexa Fluor 488- or 594-labeled donkey anti-rabbit immunoglobulin (ThermoFisher Scientific) as the secondary antibody. The Opera Phenix High

181 Content Screening System (PerkinElmer), provided by FIMM (Institute for Molecular Medicine
182 Finland) High Content Imaging and Analysis (FIMM-HCA), served for imaging of the plates stored
183 in the dark at 4°C.

184 Quantitative reverse transcription PCR (qRT-PCR)

185 qRT-PCR served for quantification of viral RNA in the cells. The primers and probe were the
186 following: forward primer 5'-GAAAGACGCGACAACCTGTGAGTC-3', reverse primer 5'-
187 GTCTAGTCCCGTTCCGGTTCTATG-3', and probe 5' 6-Fam (carboxyfluorescein)-
188 GGAGATCCGAGAGGGGAGAAGAGGAGAGGTC-BHQ (black hole quencher)-1 3', which
189 target SwSCV-1 RNA in genomic orientation. We isolated RNA for qRT-PCR using the GeneJET
190 RNA Purification Kit (ThermoFisher Scientific) with the addition of carrier RNA when purifying
191 RNA from cell culture supernatants. We used TaqMan® Fast Virus 1-Step Master Mix
192 (ThermoFisher Scientific) to set up 10 µl (half volume) reactions according to the manufacturer's
193 recommendations with the addition of 8% DMSO to prevent secondary structure formation. The
194 AriaMX real-time PCR system (Agilent) served for thermal cycling of the duplicate samples with the
195 recommended conditions: reverse transcription for 5 min at 50°C; initial denaturation for 20 s at 95°C;
196 and two amplification steps at 95°C for 3 s and 60°C for 30 s repeated for 40 cycles.

197 To generate a control RNA for copy-level quantification, we used the SwSCV-1-FWD plasmid
198 described in Szirovicza et al., 2020 (17). Briefly, FastDigest SmaI (ThermoFisher Scientific)
199 following manufacturer's protocol served for linearization of the plasmid. The GeneJet Gel
200 Purification Kit (ThermoFisher Scientific) served for purification of the linearized plasmid after
201 agarose gel separation. We used the TranscriptAid T7 High Yield Transcription Kit (ThermoFisher
202 Scientific) according to the manufacturer's protocol to *in vitro* transcribe the target RNA.
203 Subsequently, we purified the RNA by the GeneJET RNA Purification Kit (ThermoFisher Scientific),
204 diluted it into diethyl pyrocarbonate (DEPC) treated water, and stored the RNA in aliquots at -80°C
205 until use. The NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) served for quantification

206 of the control RNA and an online copy number calculator
207 (<http://endmemo.com/bio/dnacopynum.php>) for converting the concentration to RNA copies per
208 microliter. We ran the control RNA as 10-fold dilution series in duplicates for each run to generate a
209 standard curve for estimation of RNA copy numbers in cell and cell culture supernatant samples.

249 To normalize the SwSCV-1 RNA levels against a house-keeping gene, we ordered the
250 following primers and probe for the detection of *Boa constrictor* glyceraldehyde-3-phosphate
251 dehydrogenase (GAPDH): forward primer: 5' CTGGTATGACAACGAATA 3', reverse primer: 5'
252 CAGTCTTTACTCCTTAGATG 3', and probe 5' 6-Fam (carboxyfluorescein)-
253 TGAACCAACAAGTCTACCACACG -BHQ-1 3'. Reference assembly using *Python bivittatus*
254 GAPDH (GenBank accession: XM_007429612.3) as the template in Unipro UGENE (37) served to
255 obtain the mRNA for *B. constrictor* GAPDH from the reads of our earlier metatranscriptomic studies
256 (33, 38-40).

257 Near-infrared fluorescent northern blot

258 For northern blot analysis, TRIzolTM reagent (ThermoFisher Scientific) used according to the
259 manufacturer's recommendations served for isolating RNA from either T75 or T175 flasks of I/1Ki,
260 I/1Ki-2×Δ and I/1Ki-1.2×Δ. The NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) was
261 employed for quantification of the RNA solubilized into formamide after 1:100 dilution in DEPC-
262 treated water. Subsequently, we ran 3-5 μg of RNA to detect genomic SwSCV-1 RNA, 20-25 μg to
263 detect antigenomic RNA, and an ssRNA ladder (New England Biolabs) on agarose-formaldehyde
264 gels using the tricine/triethanolamine buffer system and method described by Mansour and Pestov
265 (41). Then, we transferred the RNAs from the gel to HybondTM-N+ nylon membrane (GE
266 Healthcare) by capillary transfer O/N and cross-linked the RNA to the membrane by an ultraviolet
267 cross-linker (120 mJ/cm² at 254 nm) (Analytik Jena). Incubation in prehybridization buffer (5×
268 sodium saline citrate buffer [SSC], 5× Denhardt's solution [ThermoFisher Scientific], 1% SDS, 50%
269 formamide and 100 μg ultrapure herring sperm DNA [ThermoFisher Scientific]) at 68°C or 48°C

(depending on the probe) for 2-4 h served for blocking the non-specific binding sites on the membrane. The following probes (all from Metabion International Ag): ladder-targeting (5'- IR800-AAGCAGGTCCTCGTCGCCGTACACCTCATCATACA-3'), SwSCV-1 genome-targeting (5'- IR800-GCTCTCCCCGGCAAGTCTTCTATTTCTGTCCTTCC-3'), SwSCV-1 antigenome-targeting (5'- IR800-GGAAGGACAGAAATAGAAGACTTGCCGGGGAGAGC-3'), or SDAg mRNA-targeting (5'- IR800-TAATCTCTTTTCGGTGGTAGCCTCGAGCCGCCATCC-3') diluted in prehybridization buffer served for detection. We performed the hybridization O/N at 68°C (using the genome targeting probe) or 48°C (using the antigenome and mRNA targeting probes), and washed the membrane once with 2× SSC and 0.1% SDS for 10 min at 68°C/48°C, followed by two 10 min washes with 1× SSC and 0.1% SDS at 68°C/48°C. After the washes, the Odyssey Infrared Imaging System (LI-COR Biosciences) served to record the results. The protocol was adapted from Miller et al., 2018 (42).

RESULTS

Transfection with 1.2× genome constructs initiates deltavirus replication.

In our previous study, we showed in a transfection-based assay that a plasmid bearing the SwSCV-1 genome in duplicate could initiate SwSCV-1 replication in cell culture with highest efficacy observed in bovine kidney cells (17). We hypothesized, based on RNA transfection studies with HDV (11), that a plasmid bearing a shorter 1.2× genome-length insert would suffice to initiate translation. With the idea that duplicating the antigenomic and genomic ribozymes would better facilitate replication, we ordered synthetic genes representing the 1.2× genome of SwSCV-1, TSRV-1, DabDV-1, CITV-1 and HDV-1. Figure 1 shows the organization of the synthetic blocks that we subsequently cloned in both reverse and forward orientation into a pCAGGS/MCS expression vector under the CAG promoter. Unlike in our earlier study, in which we included an additional T7 promoter in the antigenomic orientation, upstream of the DA_g to the synthetic construct (17), we did not include additional promoters that could unintentionally facilitate DA_g translation. The resulting constructs we named according to the 1.2× “deltavirus” FWD and 1.2× “deltavirus” REV scheme. The FWD constructs drive transcription of the respective deltavirus antigenome, due to which the expression or translation of SDA_g should only occur following virus replication. On the other hand, the REV constructs would generate antigenomic transcripts that would likely also mediate the SDA_g translation. To test if the shorter constructs will facilitate replication, and to estimate the cross-reactivity of our rabbit anti-SwSCV-1 DA_g antiserum, we transfected I/1Ki cells with each of the constructs. IF staining of the cells transfected with REV constructs for DA_g at 4 days post transfection (dpt) showed that the anti-SwSCV-1 DA_g antiserum clearly cross-reacted with TSRV-1 and HDV-1, but also to some extent with DabDV-1 and CITV-1 (Figure 2A). We detected DA_g in cells transfected with the FWD constructs of HDV-1, TSRV-1, and SwSCV-1, suggesting that transfection resulted in replication initiation (Figure 2B). The staining for DA_g of DabDV and CITV-1 was much less prominent on the cells transfected with FWD construct; however, the results suggest that replication

occurs also with these viruses. We also performed WB on the transfected cells and we were able to detect DAGs of SwSCV-1, HDV-1, TSRV-1 and DabDV-1 4 days after transfection with the REV constructs. However, after transfection with the FWD constructs, we were only able to detect bands for DAGs of SwSCV-1, HDV-1 and TSRV-1 (Figure 2C). The observed differences between the results of IF staining and WB could be due to the ability of the antibody to bind conformational epitopes in case of IF staining, however, in WB this is not possible.

The 1.2× and 2× SwSCV-1 genome infectious clones induce similar infection as judged by antigen expression and replication

To compare the ability of the shorter 1.2× genome length constructs to initiate replication, we transfected I/1Ki cells with 1.2× SwSCV-1 FWD and 1.2× SwSCV-1 REV and compared the antigen expression and SwSCV-1 RNA production between 1 and 5 dpt. IF staining of the transfected cells at 1-4 dpt demonstrates that both 1.2× and 2× genome constructs efficiently drive the expression of DAG (Figure 3). To better estimate the amount of DAG produced by the different constructs, we performed western blotting on samples collected between 1 and 4 dpt. The results showed that similarly to the 2× genome constructs, the transfection of 1.2× constructs led to an increasing amount of SDAG expression during the interval studied (Figure 4A). As the amount of DAG appeared to increase until 4 dpt and because the results of the IF staining suggested minute differences in the amount of DAG expression through the different construct, we performed WB analysis of samples collected at 5 dpt. The results indicate that the SDAG expression level in the cells is similar at 5 dpt, regardless of the construct used for transfection (Figure 4B). DAG expression in REV constructs could be driven by the CAG promoter of the plasmid, but since levels were similar at 5 dpt we interpret the result to suggest that DAG expression is due to replication. To add another dimension to the determination of the replication efficiency, we analyzed I/1Ki cells transfected with the 4 different constructs for SwSCV-1 RNA levels at 3 and 6 dpt by qRT-PCR. The 2× SwSCV-1 genome construct appeared to initiate replication more slowly as demonstrated by the lower amount of SwSCV-1 RNA

at 3 dpt (Figure 4C). However, at 6 dpt the cells transfected with each of the constructs showed similar SwSCV-1 RNA levels when normalized against GAPDH mRNA (Figure 4C), supporting the WB-based interpretation that all of the studied constructs can initiate replication.

Superinfection of cells transfected with 1.2× SwSCV-1 FWD construct induces infectious particle formation

To show that the 1.2× SwSCV-1 construct not only initiates virus replication in cell culture, but also induces infectious particle formation in the presence of a suitable helper virus, we superinfected 1.2× and 2× SwSCV-1 FWD transfected cells with HISV-1, a hartmanivirus demonstrated to act as a helper for SwSCV-1 (17). We titrated the supernatants collected at 3, 6 and 9 dpi with HISV-1 on clean I/1Ki cells and used supernatants collected from non-superinfected cells as the control. IF staining of cells inoculated with the supernatants at 4 dpi for SDAg served for detecting the infected cells (Figure 5A). We determined the number of infectious units by counting the fluorescent foci at each time point and the results showed I/1Ki cells transfected with 1.2× or 2× SwSCV-1 FWD constructs to be equally effective in producing infectious particles following superinfection (Figure 5B). As observed for 2× SwSCV-1 FWD in our earlier study (17), the non-superinfected 1.2× SwSCV-1 FWD cells were not able to produce infectious SwSCV-1 particles.

1.2× SwSCV-1 construct leads to persistent infection of the transfected cells

In our previous study, we showed that by maintaining I/1Ki cells after transfection with the 2× SwSCV-1 FWD construct, we could generate persistently SwSCV-1 infected cell lines (17). At the time of preparing this manuscript, we have maintained the I/1Ki-2×Δ cell line for 2.5 years, and IF staining for DA_g shows the cell line to be persistently SwSCV-1 infected (Figure 6A). To compare the replication behavior of the shorter construct further, we transfected I/1Ki cells with 1.2× SwSCV-1 FWD and continued passaging the cells. Analysis of the cells by IF staining for DA_g at 8 months post initial transfection indicates that also the 1.2× SwSCV-1 FWD construct can induce persistent infection in I/1Ki cells (Figure 6A). We compared the generated cell line, I/1Ki-1.2×Δ, to I/1Ki-2×Δ

cells further by analyzing the amount of DA_g expression using WB. The results show that DA_g expression by I/1Ki-1.2×Δ cells is at least at the level observed in I/1Ki-2×Δ cells (Figure 6B), supporting the observation of a similar replication efficiency. To further compare the cell lines, we set up a near-infrared fluorescent northern blot assay for detection of the genomic RNA, antigenomic RNA, and SDA_g mRNA. Northern blot of RNA isolated from I/1Ki-1.2×Δ and I/1Ki-2×Δ cells using a probe targeting the genomic RNA resulted in detection of a doublet band migrating at around 2.8 kilonucleotides (knt) from both cell lines (Figure 6C). To our surprise, the migration of the genomic RNA as compared to the RNA marker did not correspond to the expected 1.7-knt SwSCV-1 genomic RNA. Using higher RNA loading we detected a band migrating at around 5 knt, which likely represents the trimeric genome reported to be present in the infected cells by other researchers (43). We assume that the 2.8-knt band observed in SwSCV-1 infected cells represents the circular genome and speculate that the aberrant migration is due to the rod-like structure of the genomic RNA. We analyzed the persistently infected cells also for the presence of antigenomic RNA and succeeded in the detection when roughly 7 times more RNA was applied for the initial separation. As compared to the RNA marker, the bands detected using the antigenomic probe were approximately 3 knt by mobility (Figure 6C). In HDV the genomic RNA is approximately 5-10 times more abundant (44), and we think that, in addition to the rod-like structure, the high amount of genomic RNA further distorted the migration of the antigenomic RNA. We were unable to detect SDA_g mRNA in the persistently infected cells, but detection of the antigenomic RNA by the probe (Figure 6C) suggests that the mRNA amount was below our detection limit.

DISCUSSION

The identification of novel deltaviruses significantly divergent from HDV (14, 15, 18-21), which until 2018 was the sole representative of the previously unassigned genus *Deltavirus*, has increased the interest in deltavirus research. The identification of novel deltaviruses in various host species without traces of hepadnaviruses by others and us led to questioning the strict association of HDV and HBV. Co-incidentally, Perez-Vargas and colleagues showed that HDV is able to use helper viruses other than HBV to form infectious particles (16). We demonstrated that SwSCV-1 efficiently utilized reptarena- and hartmaniviruses as its helpers, and that the co-expression of different arena- and orthohantavirus glycoproteins can drive infectious particle formation (17). Construction of infectious clones is the first step in demonstrating that the sequences recovered through metatranscriptomic analyses are indeed complete and capable of driving replication. We reported generation of such a clone by inserting two copies of the SwSCV-1 genome in head-to-tail fashion into a mammalian expression vector, pCAGGS (17). The same approach was hence proven functional for TSRV-1 (19) as well as *Taeniopygia guttata* deltavirus and *Marmota monax* deltavirus (21). The first HDV infectious clone contained a trimeric HDV genome, and the authors utilized a dimeric genome-containing plasmid with deletion in the DA_g ORF to demonstrate the protein's role in replication (4). Constructs containing multiples of the genome make synthetic inserts longer and complicate mutational studies because each modification needs to be inserted/generated multiple times. This motivated us to attempt generation of a 1.2× genome infectious clones for initiation of deltavirus replication. The availability of tools and reagents at hand forced us to focus on comparing the replication initiation between 2× and 1.2× SwSCV-1 genome clones in depth, but we were also able to demonstrate that a similar approach might work for the recently identified deltaviruses.

The replication of HDV occurs via rolling circle replication by cellular RNA polymerases (11) during which the genomic and antigenomic ribozymes cut the produced genome multimers into unit-length pieces (45). The recently identified deltaviruses presumably share the same replication strategy and possess the genomic and antigenomic ribozymes (14, 15, 19, 21, 46). Based on the HDV literature

(47-49), we reasoned that duplicating the genomic and antigenomic ribozyme sequences would facilitate initiation of replication and/or production of unit-length genome (and antigenome). Indeed, RNA transfection studies with HDV have shown 1.2× genome copies to be most efficient in induction of virus replication (29), and a similar approach has been applied to generate HDV infectious clones (50). By applying the same principle, we constructed 1.2× genome infectious clones for HDV-1, SwSCV-1, TSRV-1, DabDV-1, and CITV-1 in both genomic and antigenomic sense, and tested the clones in I/1Ki cells, which efficiently support replication of SwSCV-1 following transfection with the 2× genome clone (17). In the REV constructs, the CAG promoter of the pCAGGS vector should mediate DA_g translation, which provides a source of DA_g for the first rounds of replication. For HDV, the reports suggest existence of an internal promoter that could drive the production of DA_g (28, 51), such a promoter would presumably act in both our REV and FWD constructs and could also contribute to replication initiation. Indeed, the comparison of DA_g production following transfection with 1.2× and 2× genome SwSCV-1 FWD and REV constructs demonstrated detectable DA_g levels to appear earlier in cells transfected with REV constructs (Figure 4A). We thus used the IF staining of DA_g from I/1Ki cells transfected with the 1.2× genome REV constructs (HDV-1, TSRV-1, DabDV-1, and CITV-1) to estimate the cross-reactivity of the anti-SwSCV-1 DA_g antiserum (15) with DA_g of the different viruses. The antiserum appeared to cross-react best with TSRV-1 DA_g that is the closest relative of SwSCV-1 from the viruses included (19). The fact that HDV-1 DA_g showed prominent nuclear staining, as would be expected based on the HDV literature, increased our confidence in the specificity of the IF staining. The antiserum appeared to cross-react moderately well with the DA_g of DabDV-1, showing mainly cytoplasmic staining. While the CITV-1 DA_g appeared to be barely detectable with the antiserum, the results did suggest production of CITV-1 DA_g. Although the expression of DA_g could come from internal promoters, we think that the DA_g produced following transfection of FWD constructs is due to initiation of replication. While the inability of our SwSCV-1 DA_g antiserum to cross-react with the DA_gs of the other viruses tested

likely explains the lower signal, it is also likely that the different deltaviruses are not replicating optimally in the *B. constrictor* cells. We hypothesize that each of the deltaviruses would be through promoter usage adapted to replicating in a specific host species.

The 1.2× genome construct design described herein could help to reduce the complexity of introducing mutations, and facilitate synthetic gene design for molecular biology studies of the recently identified deltaviruses and those that will be identified in the future. Our results with SwSCV-1 show that the 1.2× genome clone is at least as efficient as the 2× genome clone in initiation of replication. Furthermore, our results indicate that introduction of the insert in either genomic or antigenomic orientation functions equally well in the *B. constrictor* kidney cell model. Further studies with HDV-1, TSRV-1, DabDV-1, and CITV-1 in cell lines of various species could serve to demonstrate species specificity of the viruses, and to provide first evidence on the potential role of deltavirus promoters in mediating species-specific replication.

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REFERENCES

1. Rizzetto M, Canese MG, Aricò S, Crivelli O, Trepo C, Bonino F, Verme G. 1977. Immunofluorescence detection of new antigen-antibody system (delta/anti-delta) associated to hepatitis B virus in liver and in serum of HBsAg carriers. *Gut* 18:997-1003.
2. Rizzetto M, Canese MG, Gerin JL, London WT, Sly DL, Purcell RH. 1980. Transmission of the hepatitis B virus-associated delta antigen to chimpanzees. *J Infect Dis* 141:590-602.
3. Cunha C, Tavanetz JP, Gudima S. 2015. Hepatitis delta virus: A fascinating and neglected pathogen. *World J Virol* 4:313-22.
4. 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* 63:1945-1950.
5. Chen PJ, Kalpana GG, J., Mason W, Werner B, Gerin J, Taylor J. 1986. Structure and replication of the genome of the hepatitis delta virus. *Proc Natl Acad Sci USA* 83:8774-8.
6. Kos A, Dijkema R, Arnberg AC, van der Meide PH, Schellekens H. 1986. The hepatitis delta (delta) virus possesses a circular RNA. *Nature* 323:558-60.
7. Hsieh SY, Chao M, Coates L, Taylor J. 1990. Hepatitis delta virus genome replication: a polyadenylated mRNA for delta antigen. *J Virol* 64:3192-8.
8. Chao M, Hsieh SY, Taylor J. 1990. Role of two forms of hepatitis delta virus antigen: evidence for a mechanism of self-limiting genome replication. *J Virol* 64:5066-5069.
9. Luo GX, Chao M, Hsieh SY, Sureau C, Nishikura K, Taylor J. 1990. A specific base transition occurs on replicating hepatitis delta virus RNA. *J Virol* 64:1021-1027.
10. Wong SK, Lazinski DW. 2002. Replicating hepatitis delta virus RNA is edited in the nucleus by the small form of ADAR1. *Proc Natl Acad Sci USA* 99:15118-23.
11. Macnaughton TB, Shi ST, Modahl LE, Lai MM. 2002. Rolling circle replication of hepatitis delta virus RNA is carried out by two different cellular RNA polymerases. *J Virol* 76:3920-7.

- 471 12. Been MDW, G.S. 1997. Self-cleaving ribozymes of hepatitis delta virus RNA. Eur J Biochem
472 247:741-53.
- 473 13. Magnus L, Taylor J, Mason WS, Sureau C, Deny P, Norder H, Ictv Report C. 2018. ICTV
474 Virus Taxonomy Profile: Deltavirus. J Gen Virol 99:1565-1566.
- 475 14. Wille M, Netter HJ, Littlejohn M, Yuen L, Shi M, Eden JS, Klaassen M, Holmes EC, Hurt
476 AC. 2018. A Divergent Hepatitis D-Like Agent in Birds. Viruses 10:720.
- 477 15. Hetzel U, Szirovicza L, Smura T, Prahauser B, Vapalahti O, Kipar A, Hepojoki J. 2019.
478 Identification of a Novel Deltavirus in Boa Constrictors. mBio 10:e00014-19.
- 479 16. Perez-Vargas J, Amirache F, Boson B, Mialon C, Freitas N, Sureau C, Fusil F, Cosset FL.
480 2019. Enveloped viruses distinct from HBV induce dissemination of hepatitis D virus in vivo.
481 Nat Commun 10:2098.
- 482 17. Szirovicza L, Hetzel U, Kipar A, Martinez-Sobrido L, Vapalahti O, Hepojoki J. 2020. Snake
483 Deltavirus Utilizes Envelope Proteins of Different Viruses To Generate Infectious Particles.
484 mBio 11:e03250-19.
- 485 18. Chang WS, Pettersson JH, Le Lay C, Shi M, Lo N, Wille M, Eden JS, Holmes EC. 2019.
486 Novel hepatitis D-like agents in vertebrates and invertebrates. Virus Evol 5:vez021.
- 487 19. Paraskevopoulou S, Pirzer F, Goldmann N, Schmid J, Corman VM, Gottula LT, Schroeder S,
488 Rasche A, Muth D, Drexler JF, Heni AC, Eibner GJ, Page RA, Jones TC, Muller MA,
489 Sommer S, Glebe D, Drosten C. 2020. Mammalian deltavirus without hepadnavirus
490 coinfection in the neotropical rodent Proechimys semispinosus. Proc Natl Acad Sci USA
491 117:17977-17983.
- 492 20. Bergner LM, Orton RJ, Broos A, Tello C, Becker DJ, Carrera JE, Patel AH, Biek R, Streicker
493 DG. 2021. Diversification of mammalian deltaviruses by host shifting. Proc Natl Acad Sci
494 USA 118:e2019907118.

- 495 21. Iwamoto M, Shibata Y, Kawasaki J, Kojima S, Li YT, Iwami S, Muramatsu M, Wu HL, Wada
496 K, Tomonaga K, Watashi K, Horie M. 2021. Identification of novel avian and mammalian
497 deltaviruses provides new insights into deltavirus evolution. *Virus Evol* 7:veab003.
- 498 22. Edgar RC, Taylor J, Lin V, Altman T, Barbera P, Meleshko D, Lohr D, Novakovsky G,
499 Buchfink B, Basem A, Banfield JF, de la Peña M, Korobeynikov A, Chikhi R, Babaian A.
500 2020. Petabase-scale sequence alignment catalyses viral discovery. *BioRxiv* - preprint.
- 501 23. Littlejohn M, Locarnini S, Yuen L. 2016. Origins and Evolution of Hepatitis B Virus and
502 Hepatitis D Virus. *Cold Spring Harb Perspect Med* 6:a021360.
- 503 24. Perez-Vargas J, Pereira de Oliveira R, Jacquet S, Pontier D, Cosset FL, Freitas N. 2021. HDV-
504 Like Viruses. *Viruses* 13:1207.
- 505 25. Netter HJ, Barrios MH, Littlejohn M, Yuen LKW. 2021. Hepatitis Delta Virus (HDV) and
506 Delta-Like Agents: Insights Into Their Origin. *Front Microbiol* 12:652962.
- 507 26. Walker PJ, Siddell SG, Lefkowitz EJ, Mushegian AR, Adriaenssens EM, Alfenas-Zerbini P,
508 Davison AJ, Dempsey DM, Dutilh BE, Garcia ML, Harrach B, Harrison RL, Hendrickson
509 RC, Junglen S, Knowles NJ, Krupovic M, Kuhn JH, Lambert AJ, Lobočka M, Nibert ML,
510 Oksanen HM, Orton RJ, Robertson DL, Rubino L, Sabanadzovic S, Simmonds P, Smith DB,
511 Suzuki N, Van Dooerslaer K, Vandamme AM, Varsani A, Zerbini FM. 2021. Changes to virus
512 taxonomy and to the International Code of Virus Classification and Nomenclature ratified by
513 the International Committee on Taxonomy of Viruses (2021). *Arch Virol* 166:2633-2648.
- 514 27. Hepojoki J, Hetzel U, Paraskevopoulou S, Drosten C, Harrach B, Zerbini M, Koonin EV,
515 Krupovic M, Dolja V, Kuhn JH. 2020. Create one new realm (Ribozyviria) including one new
516 family (Kolmioviridae) including genus Deltavirus and seven new genera for a total of 15
517 species <https://ictv.global/ictv/proposals/2020.012D.R.Ribozyviria.zip>.
- 518 28. Tai FP, Chen PJ, Chang FJ, Chen DS. 1993. Hepatitis delta virus cDNA monomer can be used
519 in transfection experiments to initiate viral RNA replication. *Virology* 197:137-42.

- 520 29. Macnaughton TB, Lai MM. 2004. Hepatitis delta virus RNA transfection for the cell culture
521 model. *Methods Mol Med* 96:351-7.
- 522 30. Lieber A, Sandig V, Strauss M. 1993. A mutant T7 phage promoter is specifically transcribed
523 by T7-RNA polymerase in mammalian cells. *Eur J Biochem* 217:387-94.
- 524 31. Sandig V, Lieber A, Bähring S, Strauss M. 1993. A phage T7 class-III promoter functions as
525 a polymerase II promoter in mammalian cells. *Gene* 131:255-9.
- 526 32. Hetzel U, Sironen T, Laurinmaki P, Liljeroos L, Patjas A, Henttonen H, Vaheri A, Artelt A,
527 Kipar A, Butcher SJ, Vapalahti O, Hepojoki J. 2013. Isolation, identification, and
528 characterization of novel arenaviruses, the etiological agents of boid inclusion body disease.
529 *J Virol* 87:10918-35.
- 530 33. Hepojoki J, Hepojoki S, Smura T, Szirovicza L, Dervas E, Prahauser B, Nufer L, Schraner
531 EM, Vapalahti O, Kipar A, Hetzel U. 2018. Characterization of Haartman Institute snake
532 virus-1 (HISV-1) and HISV-like viruses-The representatives of genus Hartmanivirus, family
533 Arenaviridae. *PLoS Pathog* 14:e1007415.
- 534 34. Niwa H, Yamamura K, Miyazaki J. 1991. Efficient selection for high-expression transfectants
535 with a novel eukaryotic vector. *Gene* 108:193-9.
- 536 35. Korzyukov Y, Iheozor-Ejiofor R, Levanov L, Smura T, Hetzel U, Szirovicza L, de la Torre
537 JC, Martinez-Sobrido L, Kipar A, Vapalahti O, Hepojoki J. 2020. Differences in Tissue and
538 Species Tropism of Reptarenavirus Species Studied by Vesicular Stomatitis Virus
539 Pseudotypes. *Viruses* 12:395.
- 540 36. Korzyukov Y, Hetzel U, Kipar A, Vapalahti O, Hepojoki J. 2016. Generation of Anti-Boa
541 Immunoglobulin Antibodies for Serodiagnostic Applications, and Their Use to Detect Anti-
542 Reptarenavirus Antibodies in Boa Constrictor. *PLoS One* 11:e0158417.
- 543 37. Okonechnikov K, Golosova O, Fursov M, team U. 2012. Unipro UGENE: a unified
544 bioinformatics toolkit. *Bioinformatics* 28:1166-7.

- 545 38. Keller S, Hetzel U, Sironen T, Korzyukov Y, Vapalahti O, Kipar A, Hepojoki J. 2017. Co-
546 infecting Reptarenaviruses Can Be Vertically Transmitted in Boa Constrictor. PLoS Pathog
547 13:e1006179.
- 548 39. Argenta FF, Hepojoki J, Smura T, Szivovics L, Hammerschmitt ME, Driemeier D, Kipar A,
549 Hetzel U. 2020. Identification of Reptarenaviruses, Hartmaniviruses, and a Novel Chuvirus
550 in Captive Native Brazilian Boa Constrictors with Boid Inclusion Body Disease. J Virol
551 94:e00001-20.
- 552 40. Windbichler K, Michalopoulou E, Palamides P, Pesch T, Jelinek C, Vapalahti O, Kipar A,
553 Hetzel U, Hepojoki J. 2019. Antibody response in snakes with boid inclusion body disease.
554 PLoS One 14:e0221863.
- 555 41. Mansour FH, Pestov DG. 2013. Separation of long RNA by agarose-formaldehyde gel
556 electrophoresis. Anal Biochem 441:18-20.
- 557 42. Miller BR, Wei T, Fields CJ, Sheng P, Xie M. 2018. Near-infrared fluorescent northern blot.
558 RNA 24:1871-1877.
- 559 43. Wu JC, Chen PJ, Kuo MY, Lee SD, Chen DS, Ting LP. 1991. Production of hepatitis delta
560 virus and suppression of helper hepatitis B virus in a human hepatoma cell line. J Virol
561 65:1099-104.
- 562 44. Taylor JM. 2015. Hepatitis D Virus Replication. Cold Spring Harb Perspect Med 5:a021568.
- 563 45. Flores R, Grubb D, Elleuch A, Nohales MA, Delgado S, Gago S. 2011. Rolling-circle
564 replication of viroids, viroid-like satellite RNAs and hepatitis delta virus: variations on a
565 theme. RNA Biol 8:200-6.
- 566 46. de la Pena M, Ceprian R, Casey JL, Cervera A. 2021. Hepatitis delta virus-like circular RNAs
567 from diverse metazoans encode conserved hammerhead ribozymes. Virus Evol 7:veab016.

- 568 47. Kuo MY, Sharmeen L, Dinter-Gottlieb G, Taylor J. 1988. Characterization of self-cleaving
569 RNA sequences on the genome and antigenome of human hepatitis delta virus. *J Virol*
570 62:4439-44.
- 571 48. Chen PJ, Kuo MY, Chen ML, Tu SJ, Chiu MN, Wu HL, Hsu HC, Chen DS. 1990. Continuous
572 expression and replication of the hepatitis delta virus genome in Hep G2 hepatoblastoma cells
573 transfected with cloned viral DNA. *Proc Natl Acad Sci USA* 87:5253-5257.
- 574 49. Jeng KS, Daniel A, Lai MM. 1996. A pseudoknot ribozyme structure is active in vivo and
575 required for hepatitis delta virus RNA replication. *J Virol* 70:2403-10.
- 576 50. Suarez-Amaran L, Usai C, Di Scala M, Godoy C, Ni Y, Hommel M, Palomo L, Segura V,
577 Olague C, Vales A, Ruiz-Ripa A, Buti M, Salido E, Prieto J, Urban S, Rodriguez-Frias F,
578 Aldabe R, Gonzalez-Aseguinolaza G. 2017. A new HDV mouse model identifies
579 mitochondrial antiviral signaling protein (MAVS) as a key player in IFN-beta induction. *J*
580 *Hepatol* 67:669-679.
- 581 51. Macnaughton TB, Beard MR, Chao M, Gowans EJ, Lai MM. 1993. Endogenous promoters
582 can direct the transcription of hepatitis delta virus RNA from a recircularized cDNA template.
583 *Virology* 196:629-36.

FIGURE LEGENDS

Figure 1. Schematic representation of the “1.2× genome” deltavirus inserts in forward (FWD)/genomic orientation. We cloned the 1.2× genome of the displayed deltaviruses: swiss snake colony virus 1 (SwSCV-1, GenBank accession: NC_040729.1, 1.15× genome), human hepatitis D virus genotype 1 (HDV-1, M21012.1, 1.16× genome), Tome’s spiny rat virus 1 (TSRV-1, MK598005.2, 1.13× genome), Dabbling duck virus 1 (DabDV-1, NC_040845.1, 1.17× genome) and Chusan Island toad virus 1 (CITV-1, MK962760.1, 1.22× genome) into pCAGGS/MCS plasmid, both in genomic (FWD – shown in this figure) and antigenomic (REV) orientation. Each of the inserts, approximately 1.2× of the genome size, contain a single copy of the genomes flanked from each end by both the genomic and antigenomic ribozymes. The images were created using SnapGene Viewer.

Figure 2. SwSCV-1 DAg antiserum cross-reactivity with the DAg of different deltaviruses. **A)** I/1Ki cells transfected with 1.2× SwSCV-1, HDV-1, TSRV-1, DabDV-1 and CITV-1 REV constructs were stained for the DAg at 4 days post transfection using rabbit α-SwSCV-1 DAg antiserum. **B)** I/1Ki cells transfected with 1.2× SwSCV-1, HDV-1, TSRV-1, DabDV-1 and CITV-1 FWD constructs and stained for the DAg 4 days post transfection using rabbit α-SwSCV-1 DAg antiserum. Hoechst 33342 served for detection of the nuclei (left panels) and AlexaFluor 488 labelled donkey anti-rabbit IgG as the secondary antibody for DAg detection (middle panels). The right panels show overlay of the nuclear and DAg staining. The images were captured using Opera Phenix High Content Screening System (PerkinElmer) with 20× objective. **C)** I/1Ki cells transfected with 1.2× SwSCV-1, HDV-1, TSRV-1, DabDV-1 and CITV-1 REV constructs (left panel) and FWD constructs (right panel) were submitted for western blot at 4 days post transfection. The samples were separated on 4–20% Mini-PROTEAN TGX gels (Bio-Rad), transferred onto nitrocellulose, and the membranes were probed with rabbit α-SwSCV-1 DAg antiserum and affinity purified α-HDAg antibody. We loaded 1/3 volume of the 1.2× SwSCV-1 REV and FWD samples. The bands corresponding to the different

608 DAg are marked with the black rectangle. The results were recorded using Odyssey Infrared Imaging
609 System (LI-COR Biosciences).

610 **Figure 3.** Expression of DAg in I/1Ki cells following transfection with 2× and 1.2× genome
611 SwSCV-1 FWD and REV plasmids. I/1Ki cells transfected with 2× and 1.2× SwSCV-1 FWD and
612 REV plasmids were fixed and stained for the DAg using rabbit α-SwSCV-1 DAg antiserum at 1-4
613 days post transfection. AlexaFluor 488 labelled donkey anti-rabbit IgG served as the secondary
614 antibody for DAg detection. The images were captured using Opera Phenix High Content Screening
615 System (PerkinElmer) with 20× objective.

616 **Figure 4.** Western blot of I/1Ki cells after transfection with 2× and 1.2× SwSCV-1 (2×Δ and
617 1.2×Δ respectively) FWD and REV constructs. **A)** Samples of I/1Ki cells transfected with 2×Δ-FWD,
618 2×Δ-REV, 1.2×Δ-FWD, and 1.2×Δ-REV constructs collected at 1-4 days post transfection were
619 separated on 4–20% Mini-PROTEAN TGX gels (Bio-Rad), transferred onto nitrocellulose, and the
620 membranes were probed with rabbit α-SwSCV-1 DAg antiserum and mouse monoclonal anti-pan
621 actin antibody. The left panel shows 2× and the right panel 1.2× genome constructs. The results were
622 recorded using Odyssey Infrared Imaging System (LI-COR Biosciences). **B)** Samples of I/1Ki cells
623 transfected with 2×Δ-FWD, 2×Δ-REV, 1.2×Δ-FWD, and 1.2×Δ-REV constructs collected at 5 days,
624 analyzed as described in **A**. **C)** RNA isolated from I/1Ki cells transfected with 2×Δ-FWD, 2×Δ-REV,
625 1.2×Δ-FWD, and 1.2×Δ-REV constructs at 3 and 6 days post transfection were analyzed by qRT-
626 PCR targeting genomic SwSCV-1 RNA. *In vitro* transcribed RNA target served for obtaining a
627 standard curve to convert cycle threshold values into copy numbers. qRT-PCR targeting GAPDH
628 mRNA served for normalizing the results between samples. The y-axis shows copy numbers/reaction.
629 The error bars represent standard deviation.

630 **Figure 5.** Superinfection of 2× and 1.2× SwSCV-1 FWD transfected I/1Ki cells leads to
631 infectious particle production. **A)** Supernatants collected at 3, 6 and 9 days post HISV-1

632 superinfection from I/1Ki cells - transfected with 2× and 1.2× SwSCV-1 FWD constructs two weeks
 633 earlier - were used to inoculate clean I/1Ki cells. At four days post inoculation, the cells were fixed
 634 and stained using rabbit α-SwSCV-1 DAg antiserum and Alexa Fluor 488 labelled donkey anti-rabbit
 635 secondary antibody. Hoechst 33342 served for staining the nuclei. The top panels show clean I/1Ki
 636 cells infected with 100-fold diluted supernatant originating from HISV-1 superinfected 2× SwSCV-
 637 1 FWD transfected cells and the bottom panels with supernatant originating from HISV-1
 638 superinfected 1.2× SwSCV-1 FWD transfected cells. The images were captured using Opera Phenix
 639 High Content Screening System (PerkinElmer) with 20× objective. **B)** Opera Phenix High Content
 640 Screening System (PerkinElmer) served to count the number of infected cells in **A**, which enabled
 641 the quantification of infectious particles per ml of growth medium in terms of fluorescent focus
 642 forming units (FFUs – displayed on y-axis). The error bars represent standard deviation.

643 **Figure 6.** Comparison of persistently SwSCV-1 infected I/1Ki cells generated following
 644 transfection with 2× and 1.2× SwSCV-1 FWD constructs by immunofluorescence, western and
 645 northern blot. The 2×SwSCV-1 (I/1Ki-2×Δ) cell line was analyzed at approximately 2.5 years and
 646 1.2×SwSCV-1 (I/1Ki-1.2×Δ) at approximately 8 months after initial transfection, during which the
 647 cell lines were passaged at 1-2 week interval. **A)** Rabbit α-SwSCV-1 DAg antiserum and Alexa Fluor
 648 488 labelled donkey anti-rabbit secondary antibody served for IF staining of the fixed cells, and
 649 Hoechst 33342 for staining the nuclei. The top panels show staining of I/1Ki-2×Δ cells and the bottom
 650 panels the staining of I/1Ki-1.2×Δ cells. The left panels show staining of nuclei in blue, the middle
 651 panels show DAg staining in green, and the right panels show an overlay. The images were captured
 652 using Opera Phenix High Content Screening System (PerkinElmer) with 20× objective. **B)** Samples
 653 of I/1Ki-2×Δ cells and I/1Ki-1.2×Δ cells were separated on 4–20% Mini-PROTEAN TGX gels (Bio-
 654 Rad), transferred onto nitrocellulose, and the membranes probed with rabbit α-SwSCV-1 DAg
 655 antiserum and mouse monoclonal anti-pan actin antibody. The results were recorded using Odyssey
 656 Infrared Imaging System (LI-COR Biosciences). **C)** Indicated amounts of total RNA isolated from

657 I/1Ki-2×Δ cells and I/1Ki-1.2×Δ cells, and clean I/1Ki were separated on agarose gel and transferred
 658 onto nylon membrane. Probes were targeting SwSCV-1 genomic (left panel), antigenomic RNA
 659 (middle panels), SDAg mRNA (right panel), and the bands of the marker served for visualizing the
 660 RNA targets. The results were recorded using Odyssey Infrared Imaging System (LI-COR
 661 Biosciences).

Figure 1. Schematic representation of the “1.2× genome” deltavirus inserts in forward (FWD)/genomic orientation. We cloned the 1.2× genome of the displayed deltaviruses: swiss snake colony virus 1 (SwSCV-1, GenBank accession: NC_040729.1, 1.15× genome), human hepatitis D virus genotype 1 (HDV-1, M21012.1, 1.16× genome), Tome’s spiny rat virus 1 (TSRV-1, MK598005.2, 1.13× genome), Dabbling duck virus 1 (DabDV-1, NC_040845.1, 1.17× genome) and Chusan Island toad virus 1 (CITV-1, MK962760.1, 1.22× genome) into pCAGGS/MCS plasmid, both in genomic (FWD – shown in this figure) and antigenomic (REV) orientation. Each of the inserts, approximately 1.2× of the genome size, contain a single copy of the genomes flanked from each end by both the genomic and antigenomic ribozymes. The images were created using SnapGene Viewer.

Figure 2. SwSCV-1 DAG antiserum cross-reactivity with the DAG of different deltaviruses. **A)** I/1Ki cells transfected with 1.2x SwSCV-1, HDV-1, TSRV-1, DabDV-1 and CITV-1 REV constructs were stained for the DAG at 4 days post transfection using rabbit α -SwSCV-1 DAG antiserum. **B)** I/1Ki cells transfected with 1.2x SwSCV-1, HDV-1, TSRV-1, DabDV-1 and CITV-1 FWD constructs and stained for the DAG 4 days post transfection using rabbit α -SwSCV-1 DAG antiserum. Hoechst 33342 served for detection of the nuclei (left panels) and AlexaFluor 488 labelled donkey anti-rabbit IgG as the secondary antibody for DAG detection (middle panels). The right panels show overlay of the nuclear and DAG staining. The images were captured using Opera Phenix High Content Screening System (PerkinElmer) with 20x objective. **C)** I/1Ki cells transfected with 1.2x SwSCV-1, HDV-1, TSRV-1, DabDV-1 and CITV-1 REV constructs (left panel) and FWD constructs (right panel) were submitted for western blot at 4 days post transfection. The samples were separated on 4–20% Mini-PROTEAN TGX gels (Bio-Rad), transferred onto nitrocellulose, and the membranes were probed with rabbit α -SwSCV-1 DAG antiserum and affinity purified α -HDag antibody. We loaded 1/3 volume of the 1.2x SwSCV-1 REV and FWD samples. The bands corresponding to the different DAGs are marked with the black rectangle. The results were recorded using Odyssey Infrared Imaging System (LI-COR Biosciences).

Figure 3. Expression of DAst in I/1Ki cells following transfection with 2 $\times$ and 1.2 $\times$ genome SwSCV-1 FWD and REV plasmids. I/1Ki cells transfected with 2 $\times$ and 1.2 $\times$ SwSCV-1 FWD and REV plasmids were fixed and stained for the DAst using rabbit α -SwSCV-1 DAst antiserum at 1-4 days post transfection. AlexaFluor 488 labelled donkey anti-rabbit IgG served as the secondary antibody for DAst detection. The images were captured using Opera Phenix High Content Screening System (PerkinElmer) with 20 $\times$ objective.

Figure 4. Western blot of I/1Ki cells after transfection with 2 $\times$ and 1.2 $\times$ SwSCV-1 (2 $\times$ Δ and 1.2 $\times$ Δ respectively) FWD and REV constructs. **A)** Samples of I/1Ki cells transfected with 2 $\times$ Δ -FWD, 2 $\times$ Δ -REV, 1.2 $\times$ Δ -FWD, and 1.2 $\times$ Δ -REV constructs collected at 1-4 days post transfection were separated on 4–20% Mini-PROTEAN TGX gels (Bio-Rad), transferred onto nitrocellulose, and the membranes were probed with rabbit α -SwSCV-1 DA α antiserum and mouse monoclonal anti-pan actin antibody. The left panel shows 2 $\times$ and the right panel 1.2 $\times$ genome constructs. The results were recorded using Odyssey Infrared Imaging System (LI-COR Biosciences). **B)** Samples of I/1Ki cells transfected with 2 $\times$ Δ -FWD, 2 $\times$ Δ -REV, 1.2 $\times$ Δ -FWD, and 1.2 $\times$ Δ -REV constructs collected at 5 days, analyzed as described in **A**. **C)** RNA isolated from I/1Ki cells transfected with 2 $\times$ Δ -FWD, 2 $\times$ Δ -REV, 1.2 $\times$ Δ -FWD, and 1.2 $\times$ Δ -REV constructs at 3 and 6 days post transfection were analyzed by qRT-PCR targeting genomic SwSCV-1 RNA. *In vitro* transcribed RNA target served for obtaining a standard curve to convert cycle threshold values into copy numbers. qRT-PCR targeting GAPDH mRNA served for normalizing the results between samples. The y-axis shows copy numbers/reaction. The error bars represent standard deviation.

A

B

Figure 5. Superinfection of 2× and 1.2× SwSCV-1 FWD transfected I/1Ki cells leads to infectious particle production. **A)** Supernatants collected at 3, 6 and 9 days post HISV-1 superinfection from I/1Ki cells - transfected with 2× and 1.2× SwSCV-1 FWD constructs two weeks earlier - were used to inoculate clean I/1Ki cells. At four days post inoculation, the cells were fixed and stained using rabbit α -SwSCV-1 DAG antiserum and Alexa Fluor 488 labelled donkey anti-rabbit secondary antibody. Hoechst 33342 served for staining the nuclei. The top panels show clean I/1Ki cells infected with 100-fold diluted supernatant originating from HISV-1 superinfected 2× SwSCV-1 FWD transfected cells and the bottom panels with supernatant originating from HISV-1 superinfected 1.2× SwSCV-1 FWD transfected cells. The images were captured using Opera Phenix High Content Screening System (PerkinElmer) with 20× objective. **B)** Opera Phenix High Content Screening System (PerkinElmer) served to count the number of infected cells in **A**, which enabled the quantification of infectious particles per ml of growth medium in terms of fluorescent focus forming units (FFFUs – displayed on y-axis). The error bars represent standard deviation.

Figure 6. Comparison of persistently SwSCV-1 infected I/1Ki cells generated following transfection with 2 $\times$ and 1.2 $\times$ SwSCV-1 FWD constructs by immunofluorescence, western and northern blot. The 2 $\times$ SwSCV-1 (I/1Ki-2 $\times$ Δ) cell line was analyzed at approximately 2.5 years and 1.2 $\times$ SwSCV-1 (I/1Ki-1.2 $\times$ Δ) at approximately 8 months after initial transfection, during which the cell lines were passaged at 1-2 week interval. **A)** Rabbit α -SwSCV-1 DAG antiserum and Alexa Fluor 488 labelled donkey anti-rabbit secondary antibody served for IF staining of the fixed cells, and Hoechst 33342 for staining the nuclei. The top panels show staining of I/1Ki-2 $\times$ Δ cells and the bottom panels the staining of I/1Ki-1.2 $\times$ Δ cells. The left panels show staining of nuclei in blue, the middle panels show DAG staining in green, and the right panels show an overlay. The images were captured using Opera Phenix High Content Screening System (PerkinElmer) with 20 $\times$ objective. **B)** Samples of I/1Ki-2 $\times$ Δ cells and I/1Ki-1.2 $\times$ Δ cells were separated on 4–20% Mini-PROTEAN TGX gels (Bio-Rad), transferred onto nitrocellulose, and the membranes probed with rabbit α -SwSCV-1 DAG antiserum and mouse monoclonal anti-pan actin antibody. The results were recorded using Odyssey Infrared Imaging System (LI-COR Biosciences). **C)** Indicated amounts of total RNA isolated from I/1Ki-2 $\times$ Δ cells and I/1Ki-1.2 $\times$ Δ cells, and clean I/1Ki were separated on agarose gel and transferred onto nylon membrane. Probes were targeting SwSCV-1 genomic (left panel), antigenomic RNA (middle panels), SDag mRNA (right panel), and the bands of the marker served for visualizing the RNA targets. The results were recorded using Odyssey Infrared Imaging System (LI-COR Biosciences).