Functional Properties and Genetic Relatedness of the Fusion and Hemagglutinin-Neuraminidase Proteins of a Mumps Virus-Like Bat Virus

Nadine Krüger,^a Markus Hoffmann,^{a*} Jan Felix Drexler,^b Marcel Alexander Müller,^b Victor Max Corman,^b Christian Sauder,^c Steven Rubin,^c Biao He,^d Claes Örvell,^e Christian Drosten,^b Georg Herrler^a

Institute of Virology, University of Veterinary Medicine Hannover, Hannover, Germany^a; Institute of Virology, University of Bonn Medical Centre, Bonn, Germany^b; Division of Viral Products, Center for Biologics Evaluation and Research, FDA, Bethesda, Maryland, USA^c; Department of Infectious Diseases, University of Georgia, Athens, Georgia, USA^d; Division of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden^e

ABSTRACT

A bat virus with high phylogenetic relatedness to human mumps virus (MuV) was identified recently at the nucleic acid level. We analyzed the functional activities of the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins of the bat virus (batMuV) and compared them to the respective proteins of a human isolate. Transfected cells expressing the F and HN proteins of batMuV were recognized by antibodies directed against these proteins of human MuV, indicating that both viruses are serologically related. Fusion, hemadsorption, and neuraminidase activities were demonstrated for batMuV, and either bat-derived protein could substitute for its human MuV counterpart in inducing syncytium formation when coexpressed in different mammalian cell lines, including chiropteran cells. Cells expressing batMuV glycoproteins were shown to have lower neuraminidase activity. The syncytia were smaller, and they were present in lower numbers than those observed after coexpression of the corresponding glycoproteins of a clinical isolate of MuV (hMuV). The phenotypic differences in the neuraminidase and fusion activity between the glycoproteins of batMuV and hMuV are explained by differences in the expression level of the HN and F proteins of the two viruses. In the case of the F protein, analysis of chimeric proteins revealed that the signal peptide of the bat MuV fusion protein is responsible for the lower surface expression. These results indicate that the surface glycoproteins of batMuV are serologically and functionally related to those of hMuV, raising the possibility of bats as a reservoir for interspecies transmission.

IMPORTANCE

The recently described MuV-like bat virus is unique among other recently identified human-like bat-associated viruses because of its high sequence homology (approximately 90% in most genes) to its human counterpart. Although it is not known if humans can be infected by batMuV, the antigenic relatedness between the bat and human forms of the virus suggests that humans carrying neutralizing antibodies against MuV are protected from infection by batMuV. The close functional relationship between MuV and batMuV is demonstrated by cooperation of the respective HN and F proteins to induce syncytium formation in heterologous expression studies. An interesting feature of the glycoproteins of batMuV is the downregulation of the fusion activity by the signal peptide of F, which has not been reported for other paramyxoviruses. These results are important contributions for risk assessment and for a better understanding of the replication strategy of batMuV.

Mumps virus (MuV) belongs to the genus *Rubulavirus* within the *Paramyxoviridae* family. Mumps viruses are divided into 12 genotypes based on genetic variations of the small hydrophobic (SH) protein gene (1–5).

Mumps is a highly contagious disease with mild symptoms, such as fever, headache, and uni- or bilateral parotitis, which is the hallmark of the disease and occurs in 90% of all clinical cases (6). In rare events, mumps can result in complications like meningitis or orchitis (7, 8). So far, humans are the only known host of MuV.

Recently, the detection of genomic RNA of an MuV-related paramyxovirus (BatPV/Epo_spe/AR1/DCR/2009; batMuV; GenBank accession number [HQ660095](https://www.ncbi.nlm.nih.gov/nuccore/HQ660095)), in an African flying fox of the genus *Epomophorus* (Epaulet fruit bat) in 2009 in the Democratic Republic of Congo, has been reported (9). This virus (batMuV) shared more than 90% amino acid homology in most of its genes with MuV. Polyclonal antibodies from bat sera cross-reacted with MuV proteins (9), suggesting a close genetic and antigenic relatedness between MuV and batMuV. In addition to batMuV, several other bat paramyxoviruses related to MuV and other mammalian rubulaviruses were detected, suggesting a bat origin of

MuV and the whole paramyxovirus genus *Rubulavirus* (9). So far, all efforts to isolate a batMuV have failed, which makes it difficult to evaluate the zoonotic potential of these viruses.

The infection of cells by MuV is initiated by the binding of the hemagglutinin-neuraminidase glycoprotein (HN), a type II mem-

Received 29 December 2014 Accepted 28 January 2015

Accepted manuscript posted online 4 March 2015

Citation Krüger N, Hoffmann M, Drexler JF, Müller MA, Corman VM, Sauder C, Rubin S, He B, Örvell C, Drosten C, Herrler G. 2015. Functional properties and genetic relatedness of the fusion and hemagglutinin-neuraminidase proteins of a mumps virus-like bat virus. *J Virol* 89:4539–4548. doi:10.1128/JVI.03693-14.

Editor: D. S. Lyles

Address correspondence to Georg Herrler, Georg.Herrler@tiho-hannover.de.

* Present address: Markus Hoffmann, Unit of Infection Models, German Primate Center, Göttingen, Germany.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.03693-14

brane protein, to sialic acids of cell surface macromolecules (10). MuV has been shown to bind to erythrocytes from different mammalian and avian species (11–15), but the binding activity of MuV HN has not been studied in detail. Sialic acids present in α 2-3, α 2-6, and/or α 2-8 linkages may serve as receptor determinants for MuV. The affinity of the HN interaction with sialic acids varies depending on the strain used (16–19).

The release of the viral genome into the cytoplasm is mediated by the action of the MuV fusion protein (F), which induces the fusion of the viral envelope with the plasma membrane of the target cell. Coexpression of F and HN on the surface of infected cells results in the fusion of neighboring cells and in the formation of multinucleated giant cells, so-called syncytia (20, 21). The ability to fuse cells correlates with the neuraminidase (NA) activity of the MuV HN (20, 21), suggesting that a high level of neuraminidase activity promotes a rapid release of virions without the occurrence of cell-to-cell fusion, whereas low neuraminidase activity supports the extracellular association of virions with infected cells, thereby increasing the likelihood of cell-to-cell fusion (22). Sequence analysis of MuV F suggests that the multibasic amino acid sequence R-R-H-K-R directly upstream of the fusion peptide represents the cleavage site of the F protein (23), and that the proteolytic activation is induced by furin-like enzymes (24).

The amino acid sequences of the batMuV F and HN proteins are closely related to the glycoproteins from human MuV strains. To address the question of whether these surface glycoproteins are functionally related to their human counterparts, the open reading frames (ORFs) were cloned into expression plasmids. The viral proteins synthesized in transfected cells were analyzed for functional activity.

We report that the surface glycoproteins F and HN of batMuV are able to mediate syncytium formation following coexpression, even after heterologous coexpression with either of the glycoproteins of a human isolate of MuV. The phenotype of the syncytia (size and number) is determined by the signal peptides of the respective F proteins, as indicated by expression studies with chimeric F proteins. Furthermore, batMuV HN was found to have sialic acid-binding and neuraminidase activity.

(Part of this work was performed by N.K. in partial fulfillment of the requirements for a doctoral degree from Stiftung Tierärztliche Hochschule Hannover.)

MATERIALS AND METHODS

Sequence homology analysis. For the analysis of the sequence homology on the amino acid level of the batMuV and hMuV (a clinical isolate of MuV) F proteins to those of known MuV isolates, first a consensus sequence for the F protein of a total of 26 different MuV isolates (published in the NCBI database; GenBank accession numbers EU884413.1, FJ556896.1, AF467767.2, GU980052.1, AF338106, AY681495.1, AY508995, AB600843.1, JX287386.1, EU370206.3, JX287388.1, AY309060.1, AB600942.1, AF314561.1, AF314558.1, AB823535.1, JN012242.1, KF481689.1, AF280799.1, EU370207.1, JN635498.1, JX287385.1, JX287391.1, JX287390.1, JX287387.1, and JX287389.1) was created. In addition, and to take the intervariability of the 26 MuV isolates into account, the conservation between the individual MuV isolates was analyzed using the PProfile ALIgment (PRALINE) software (<http://www.ibi.vu.nl/programs/praline/>) (25, 26). The batMuV and hMuV F proteins next were compared to the consensus sequence, again using the PRALINE software. The readout was interpreted as follows. A conservation score of 0 for an amino acid residue (aa) at a certain position was considered a conservation level of 0%, while a

conservation score of 10 indicated a conservation level of 100% for the respective aa.

To compare different functional domains within the MuV F in addition to the full-length protein (aa 1 to 538), the mean conservation levels of F₁ (aa 103 to 538) and F₂ (aa 1 to 102) subunits, the whole ectodomain (ED; aa 1 to 485), the transmembrane domain (TD; aa 486 to 510), the cytoplasmic tail (CT; aa 511 to 538), and the signal peptide (SP; aa 1 to 20) were calculated.

Cells. BHK-21, Vero76, and HeLa cells (kindly provided by A. Mäisner) were maintained in Dulbecco's minimum essential medium (DMEM; Gibco) supplemented with 5% fetal calf serum (FCS; Biochrom). Chiropteran cells derived from different species and organs were used in this study: HypNi/1.1 (*Hypsiphys monstrosus* kidney cells), a clonal cell line produced from a mixed culture (HypNi/1) (27), HypLu/2 (*Hypsiphys monstrosus* lung cells, mixed culture) (28), and EidNi/41 cells (*Eidolon helvum* kidney cells, mixed culture) (29) were maintained in DMEM containing 10% FCS. All cells were cultivated in 75-cm² tissue culture flasks (Greiner Bio-One) at 37°C and 5% CO₂.

Expression plasmids. Expression plasmids were cloned from cDNA of the ORFs of batMuV F and HN (GenBank accession number HQ660095.1) using the following primers: batMuV F forward (for), AAGGCCGGATC CATGAGAAAACTCTAGCT; batMuV F reverse (rev), AAGGCCGTC GACTTAGTATCTCACAAGGTC; batMuV HN for, AAGGCCGGATCC ATGGAGCCATCAAAATTA; and batMuV HN rev, AAGGCCGTCGAC TCATGTGATAGTCAATCT. The DNA was cloned into the expression vector pCG1 (30), kindly provided by R. Cattaneo, using the introduced BamHI and SalI restriction sites (underlined above). The ORFs of the respective proteins of MuV were cloned from the cDNA of an MuV isolate (hMuV) that was isolated from a hospitalized patient at the University of Bonn Medical Centre (GenBank accession numbers KM519599 [hMuV F] and KM519600 [hMuV HN]) using the following primers: hMuV F for, AAGGCCGGATCCATGAAGGCTTTTTCAG; hMuV F rev, AAGCCG TCGACTTAGTACCTGATGAGATC; hMuV HN for, AAGGCCGGATC CATGGAACCTCGAAACTC; and hMuV HN rev, AAGGCCGTCGAC TTAGGTAATGGTCAGGCGGG. In addition, chimeric MuV fusion proteins with an exchange of the F₁ subunit (batMuV F₂; batMuV F, aa 1 to 102; hMuV F, aa 103 to 538; hMuV F₂; hMuV F, aa 1 to 102; batMuV F, aa 103 to 538) or the predicted signal peptide (SP; amino acids 1 to 20) were generated (hMuV F SP, hMuV F aa 1 to 20 and batMuV F aa 21 to 538; batMuV F SP, batMuV F aa 1 to 20 and hMuV F aa 21 to 538) by hybridization PCR (sequences for hybridization primers are available upon request). Furthermore, epitope-labeled constructs which contain a hemagglutinin (HA) epitope between the aa residues 96 and 97 were generated for both MuV F proteins (hMuV F-HA and batMuV F-HA) as well as for the MuV F SP chimeras (hMuV F SP-HA and batMuV F SP-HA).

Furthermore, the pEGFP-N1 vector (Clontech) was used for transfection.

Transfection. The transfection of the different mammalian cell lines was performed by two different methods. For immunofluorescence analysis as well as the hemadsorption, qualitative neuraminidase, and fusion assays, Lipofectamine 2000 transfection reagent (Life Technologies) was used according to the manufacturer's protocol. Since this transfection method resulted in high background levels in flow cytometry analysis and the quantitative neuraminidase assay, here, the ICAfectin 441 transfection reagent (In-Cell-Art) was used, also according to the manufacturer's protocol.

IFA. Cells were grown on coverslips and transfected for the expression of MuV glycoproteins. At 24 h posttransfection (p.t.), cells were fixed with phosphate-buffered saline (PBS)–3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and subsequently incubated with either of the following primary antibodies (detailed information is in Fig. 1): (i) monoclonal antibodies directed against the F protein of the Enders strain (antibodies F-2117 and F-5418) or the Iowa.US/2006[G] strain (antibody IA-F) of MuV (both raised in mice), or (ii) antibodies directed against the cytoplasmic tail of the F protein or the HN protein [designated anti-

FIG 1 Expression of human- and bat-derived MuV F and HN. HypNi/1.1 cells were transfected with the empty vector (pCG1) or with expression plasmids for human- or bat-derived MuV F (rows a to d) or HN (row e). The proteins were detected in permeabilized cells using MuV-specific primary antibodies: (row a) anti-mumps F 2117, (row b) anti-mumps F 5418, (row c) anti-mumps IA F, (row d) anti-mumps F (CT), and (row e) anti-mumps HN (CT), followed by incubation with Cy3-labeled secondary antibodies. The scale bar indicates 25 μ m.

mumps F(CT) or anti-mumps HN(CT), respectively; both raised in rabbit]. Cy3-conjugated antibodies directed against rabbit or mouse IgG F(ab')₂ fragment (both purchased from Sigma-Aldrich) served as secondary antibodies. Incubation with primary or secondary antibodies was performed in humidity chambers for 1 h or 30 min, respectively, and after each incubation step the coverslips were washed three times with PBS to remove residual antibodies. Finally, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI; Roth) and mounted in Mowiol (Calbiochem) containing 2.5% DABCO (1,4-diazabicyclo [2.2.2]octane; Sigma-Aldrich). Immunofluorescence analysis (IFA) was performed using a Nikon Eclipse Ti microscope and NIS Elements AR software (Nikon).

To analyze the intracellular and surface expression of the HA epitope-labeled MuV F constructs, cells were transfected for the expression of hMuV F-HA, batMuV F-HA, hMuV F SP-HA, or batMuV F SP-HA. At 24 h p.t., cells were fixed and incubated with an anti-HA antibody raised in rabbit (Sigma-Aldrich) for 1 h. Afterwards, cells were permeabilized and incubated with a second anti-HA antibody which had been raised in

mouse (Sigma-Aldrich) for 1 h. Subsequently, the cells were incubated with Alexa Flour 488 goat anti-rabbit and Alexa Flour 568 goat anti-mouse antibodies (both purchased from Life Technologies). Antibody incubation, mounting, and microscopic examination were performed as described before.

To quantify the surface expression of hMuV F, batMuV F, hMuV F SP, and batMuV F SP, cells were cotransfected with the HA epitope-labeled constructs and the pEGFP-N1 vector (Clontech) to evaluate transfection efficiency. The cells next were transferred to reaction tubes and incubated with antibodies as described for the flow cytometry analysis.

The cells next were fixed by incubation with PBS-3% paraformaldehyde for 20 min. Finally, the cells were washed again to remove residual paraformaldehyde and resuspended in 100 μ l PBS without calcium (PBSM)-1% bovine serum albumin (BSA)-3 mM EDTA. Ten μ l of the cell suspension was transferred into a Neubauer improved counting chamber (Roth). The total cell numbers, as well as the numbers of GFP-positive cells, were counted for each sample using the ImageJ software to account for differences in transfection efficiency. The surface expression of the different MuV F constructs was analyzed by counting the phycoerythrin (PE)-positive cells. This experiment was performed three times, with each value determined in triplicate.

In order to analyze the surface expression of the MuV HN proteins, pCG1-, hMuV HN-, or batMuV HN-transfected cells were incubated with a polyclonal antibody directed against the HN protein of the SBL-1 strain (rabbit) (31), without prior permeabilization, followed by incubation with Alexa Flour 568 goat anti-rabbit antibodies (Life Technologies).

Flow cytometry analysis. BHK-21 cells were grown in 10-cm dishes (Greiner Bio-One) and transfected with the empty vector pCG1 or with hMuV F-HA, batMuV F-HA, hMuV F SP-HA, or batMuV F SP-HA. At 24 h p.t., cells were washed and detached from the bottom of the plates using a cell scraper. The cells next were transferred to reaction tubes, and all future washing steps were performed as follows. First, the cells were collected by centrifugation at 600 $\times$ g and 4°C for 5 min, and the pellet was resuspended in PBSM-1% BSA-3 mM EDTA. This washing step was repeated three times before the cells were resuspended in PBSM-1% BSA containing anti-HA antibody (rabbit; 1:500; Sigma-Aldrich) and incubated for 1 h on an overhead shaker at 4°C. Afterwards, the cells were washed and resuspended in PBSM-1% BSA containing a biotin-conjugated anti-rabbit antibody (1:1,000; Sigma-Aldrich), again for 1 h at 4°C on an overhead shaker. After an additional washing interval, the cells were resuspended in PBSM-1% BSA containing PE-conjugated streptavidin (1:1,000; Bio-Rad) for fluorescence labeling and incubated for 30 min at 4°C on an overhead shaker. The cells next were washed and fixed by incubation with PBS-3% paraformaldehyde for 20 min. Finally, the cells were washed again to remove residual paraformaldehyde and resuspended in 500 μ l PBSM-1% BSA-3 mM EDTA, and flow cytometry was performed using a Beckman Coulter Epics XL and the Expo 32 ADC XL4 Color software (Beckman Coulter). The experiment was performed three times with each value determined in quadruplicate.

Hemadsorption assay. HypNi/1.1 cells were grown in 24-well plates and transfected with either plasmids for expression of HN from hMuV or batMuV, respectively, or with the empty vector pCG1. At 18 h p.t., the medium was removed and the cells were washed carefully with PBS three times, followed by incubation with 250 μ l PBS containing 2% chicken erythrocytes (kindly provided by S. Rautenschlein, Clinic for Poultry, University of Veterinary Medicine Hannover) per well for 10 min at 4°C. To analyze the role of sialic acids for HN interaction, this experiment was performed in parallel with erythrocytes which had been preincubated with NA from *Clostridium perfringens* (0.1 μ U/ μ l; Sigma-Aldrich) at 37°C for 30 min. Afterwards, the cells were washed five times with PBS to remove unbound erythrocytes. Finally, the cells were fixed with PBS-3% paraformaldehyde and analyzed via light microscopy. The assays were performed at least five times.

Neuraminidase activity assay. (i) Qualitative neuraminidase assay. HypNi/1.1 cells were transfected with hMuV HN, batMuV HN, or

batMuV H9 (A/chicken/Emirates/R66/2002, H9N2; kindly provided by Jürgen Stech). Chicken erythrocytes were added to the transfected cells as mentioned in the description of the hemadsorption assay. The cells next were washed five times with PBS and further incubated at 37°C for 30 min at 1-h intervals. After further washing with PBS, the cells were fixed with PBS–3% paraformaldehyde and analyzed by light microscopy. The assay was performed at least five times.

(ii) Quantitative neuraminidase activity assay. 2'-(4-Methylumbelliferyl)- α -D-N-acetylneuraminic acid (4-MUNANA; Sigma-Aldrich) was used to measure the neuraminidase activity of the HN proteins. HypNi/1.1 cells were grown in black 96-well plates and transfected with MuV glycoproteins or the empty expression plasmid pCG1. At 24 h p.t., the medium was removed and cells were washed three times with cold PBS. Transfected cells were incubated with 0.3 mM 4-MUNANA at 37°C for 1 h. As a control, *C. perfringens* neuraminidase (Sigma-Aldrich) was added to nontransfected cells at different concentrations (5.0 mU and 10.0 mU) and incubated as described for the transfected cells. The reaction was stopped by the addition of a stop solution containing 0.5 M NaOH and 25% ethanol (32). Neuraminidase activity was measured by a GENios pro chemiluminometer (Tecan) using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The assay was performed at least five times, with each value determined in quadruplicate.

Fusion assay. Cells were grown in 24-well plates and cotransfected for the expression of human- or bat-derived MuV F and HN proteins, as well as chimeric proteins. At 12 h p.t., the medium was removed, and the cells were washed three times with PBS and fixed by incubation with ice-cold methanol-acetone (1:1, vol/vol) for 30 s. Subsequently, the cells were washed with PBS twice and incubated with May Grünwald solution (Sigma-Aldrich) for 5 min, followed by washing with PBS and incubation with Giemsa staining solution (1:10 diluted with double-distilled water; Sigma-Aldrich) for 20 min. Finally, the cells were washed with aqua bidest, air dried, and analyzed via light microscopy for the presence of syncytia.

To analyze the coexpression of the hMuV and batMuV glycoproteins by IFA, permeabilized cells first were incubated with a mixture of antibodies directed against the F protein of the Enders strain (F-5418 mouse) and the HN protein of the SBL-1 strain (rabbit), followed by incubation with Alexa Flour 488 goat anti-mouse IgG and Alexa Flour 568 goat anti-rabbit IgG (both Life Technologies). Subsequently, staining of the nuclei, mounting, and microscopic analysis were performed as mentioned before. Only syncytia with a minimum of five nuclei were considered, and the assay was performed at least five times.

RESULTS

Expression of bat-derived MuV F and HN glycoproteins in mammalian cells. To analyze the F and HN proteins of batMuV, HypNi/1.1 cells and Vero76 cells were transfected with the respective expression plasmids. The former cell line recently has been shown to support the functional activity of glycoproteins derived from a bat-borne African henipavirus (33). To visualize the glycoproteins, different available antibodies were used for immunostaining. As shown in Fig. 1 for HypNi/1.1 cells, three monoclonal antibodies directed against the F protein of the Enders strain (34) or the Iowa.US/2006 strain recognized the F proteins of both the hMuV isolate used in our study and the batMuV. A rabbit antiserum directed against the cytoplasmic tail of MuV F also recognized both F proteins. In addition, the rabbit antiserum directed against the cytoplasmic tail of HN could be used to visualize HN from both hMuV and batMuV. A similar result was obtained with Vero76 cells (data not shown). This result indicates that the F proteins of hMuV and batMuV are serologically related, confirming preliminary data obtained with polyclonal sera (9).

Hemadsorption activity of batMuV HN proteins. To get information about the functional activities of the glycoproteins of

FIG 2 Hemadsorption of chicken erythrocytes to transfected HypNi/1.1 cells. (A) HypNi/1.1 cells were transfected with the empty vector or expression plasmids for either hMuV HN or batMuV HN. At 18 h p.t., the cells were incubated at 4°C with chicken erythrocytes that either were left untreated (–NA) or that had been pretreated with *C. perfringens* neuraminidase (+NA). Cells were analyzed for bound erythrocytes by light microscopy. The scale bar indicates 25 μm. (B) Transfected HypNi/1.1 cells expressing influenza virus hemagglutinin of the subtype H9, hMuV HN, or batMuV HN were incubated with chicken erythrocytes at 4°C, followed by further incubation at 37°C for 30 min or 1 h. The scale bar indicates 50 μm.

batMuV, the HN protein was analyzed for sialic acid binding activity. A convenient way to determine the interaction of viruses with sialic acids is a hemagglutination assay. As no virus particles were available for batMuV, we performed a hemadsorption assay with cells expressing the HN protein on their surface. Transfected HypNi/1.1 cells expressing either of the HN proteins were incubated with a 2% chicken erythrocyte suspension. No hemadsorption was observed with control cells transfected with an empty vector. In contrast, cells expressing the HN proteins of either hMuV or batMuV, bound erythrocytes that could not be washed away (Fig. 2A). The binding of the erythrocytes to the HN-expressing cells was sialic acid dependent, as no hemadsorption activity was detectable with erythrocytes that had been pretreated with neuraminidase. This result indicates that the HN protein of batMuV, like its counterpart from hMuV, has sialic acid binding activity.

Neuraminidase activity. Apart from binding to sialylated sialoglycoconjugates, the HN protein of paramyxoviruses also is known for its neuraminidase activity. To analyze the HN protein of batMuV for its ability to cleave sialic acids, transfected cells were subjected to a hemadsorption assay. After the erythrocytes had bound to the cells, the samples were incubated at 37°C to enable

FIG 3 Neuraminidase activity of hMuV and batMuV HN proteins at the cell surface. (A) Transfected HypNi/1.1 cells as well as different dilutions of purified *C. perfringens* NA were incubated with 4-MUNANA. The emission at a wavelength of 460 nm was measured after excitation at a wavelength of 355 nm and is given in relative fluorescence units (RFU). (B) BHK-21 cells were transfected with the empty vector (pCG1), hMuV HN, or batMuV HN. HN that was expressed on the cell surface was detected by subsequent incubation with an antibody directed against the SBL-1 strain HN protein and an Alexa Fluor 568 anti-rabbit antibody. The insets show nuclei visualized by DAPI staining.

the neuraminidase of the HN proteins to cleave the surface-bound sialic acids and to release the bound erythrocytes into the supernatant. As shown in Fig. 2B, cells expressing either of the MuV proteins showed a decreased binding of erythrocytes when incubated for 30 min at 37°C. One hour after the temperature shift, no bound erythrocytes were detectable. For comparison, an influenza virus HA of the subtype H9 was included as a control. Cells expressing the H9 protein bound erythrocytes efficiently. As this protein exhibits no neuraminidase activity, incubation for 1 h at 37°C did not result in the release of erythrocytes (Fig. 2B).

Since this result was obtained with a qualitative assay, we wanted to confirm the neuraminidase activity of batMuV by a quantitative assay. For this purpose, transfected HypNi/1.1 cells expressing the HN protein of either hMuV or batMuV were incubated with 4-MUNANA. This substrate is cleaved by the enzymatic activity of neuraminidases, resulting in a fluorescence signal after the release of sialic acid. Incubation of transfected HypNi/1.1 cells expressing the HN protein of hMuV exhibited an enzyme activity similar to that observed when the substrate was incubated with 10 μ U of purified neuraminidase from *C. perfringens* (Fig. 3A). The enzyme activity of the batMuV HN protein was lower than that of hMuV HN. No fluorescence signals above background levels were detectable for cells expressing no foreign protein (empty vector). It should be noted that the choice of transfection reagent was critical for detection of the enzyme activity of batMuV HN. In contrast to the reagent ICAfectin 441, which was used for the result shown in Fig. 3, transfection by Lipofectamine

2000 resulted in high background levels that allowed detection of neuraminidase activity only with the HN of hMuV but not with that of batMuV. To address the question of whether differences in the level of surface expression are responsible for the difference in the enzyme activity between the two HN proteins, nonpermeabilized cells were incubated with polyclonal antibodies directed against the HN protein of the SBL-1 strain of MuV to detect only the HN proteins which are expressed on the cell surface. By this approach, a stronger surface expression was detected with hMuV HN than with the batMuV HN (Fig. 3B), suggesting that a lower surface expression level rather than reduced neuraminidase activity of batMuV HN is responsible for the discrepancy between the two HN proteins.

Coexpression of batMuV F and HN results in syncytium formation in different mammalian cell lines. Coexpression of F and HN glycoproteins is required for cell-to-cell fusion, resulting in the formation of multinucleated giant cells. To analyze the glycoproteins of batMuV for fusion activity, chiropteran (HypNi/1.1, HypLu/2, and EidNi/41), human (HeLa), simian (Vero76), and rodent (BHK-21) cells were cotransfected to express the batMuV F and HN proteins and screened for the presence of syncytia at 12 h p.t. Only giant cells with a minimum of five nuclei were considered syncytia. In addition, the expression levels of the F and HN proteins were analyzed by IFA using antibodies directed against MuV F (anti-mumps F-5418) or HN (anti-mumps HN[CT]) with their respective secondary, fluorescence-labeled antibodies. Compared to their respective hMuV counterparts, both F and HN of batMuV showed lower fluorescence intensity, as well as a lower number of F or HN protein-expressing cells (Fig. 4A). As shown for HypNi/1.1 cells, the single expression of hMuV and batMuV F or HN proteins did not mediate cell-to-cell fusion (Fig. 4A). Syncytia were obtained only in cells coexpressing F and HN proteins. This result was obtained for all cell lines tested. Due to the weak fluorescence signal of batMuV F and HN proteins, especially in syncytia, cotransfected cells were stained with May Grünwald and Giemsa staining solution to visualize syncytium formation.

Coexpression of F and HN proteins from hMuV resulted in the formation of huge syncytia in all cell lines analyzed (Fig. 4B). The batMuV glycoproteins also were able to mediate fusion in all chiropteran and nonchiropteran cell lines. However, the size of the syncytia was notably smaller than the sizes of those observed after the coexpression of the hMuV glycoproteins (Fig. 4B). Also, the size of the syncytia induced by the batMuV glycoproteins did not increase significantly at later times p.t. (data not shown). Cells expressing the glycoproteins of hMuV could not be analyzed at later time points because of the cytopathic effect resulting in cell detachment.

Human- and bat-derived MuV glycoproteins cooperate in syncytium formation. After having shown that the glycoproteins of batMuV are able to induce the formation of syncytia, a heterologous fusion assay was performed to find out whether human- and bat-derived MuV glycoproteins are able to interact with each other. Coexpression of hMuV F and batMuV HN as well as the coexpression of batMuV F and hMuV HN induced syncytium formation (Fig. 4C). Syncytia obtained from the heterologous expression were smaller than those observed after homologous expression of hMuV F and HN proteins and reflect the phenotype induced by coexpression of the two batMuV proteins.

The signal peptide of the MuV fusion proteins determines the size of the syncytia. The amino acid sequence of batMuV F

FIG 4 Homologous (A and B) and heterologous (C) coexpression of human- or bat-derived MuV F and HN proteins. (A) HypNi/1.1 cells were transfected for the single expression of hMuV F, hMuV HN, batMuV F, or batMuV HN or for the coexpression of hMuV F and HN or batMuV F and HN. MuV F proteins were detected by subsequent incubation with anti-MuV F-5418 and Alexa Flour 488 goat anti-mouse antibodies, whereas MuV HN proteins were detected by incubation with anti-MuV HN(CT) and Alexa Flour 568 goat anti-rabbit antibodies. The scale bar indicates 25 μ m. (B) Cells were transfected with the empty vector pCG1, hMuV F and HN, or batMuV F and HN. At 12 h p.t., the cells were fixed with methanol-acetone (1:1) and incubated in May Grünwald and Giemsa staining solution. The scale bar indicates 25 μ m. (C) BHK-21 cells were grown on coverslips and transfected for the coexpression of hMuV F and hMuV (upper left), batMuV HN (upper right), batMuV F and hMuV (lower left), or batMuV HN (lower right). At 12 h p.t., the cells were fixed with methanol-acetone (1:1) and incubated in May Grünwald and Giemsa staining solution. The scale bar indicates 25 μ m.

TABLE 1 Sequence homology between the F proteins of MuV and batMuV

Domain	Conservation of MuV F within human strains (%)	Conservation level to the consensus sequence ^a (%) for:	
		hMuV F ^b	batMuV F
Full-length F	97.7	98.9	94.6
F ₁ subunit	98.3	99.2	96.5
F ₂ subunit	95.3	97.4	86.5
Signal peptide ^c	86.5	96.0	69.0
Ectodomain	97.7	98.8	94.3
Transmembrane domain	96.0	99.2	96.8
Cytoplasmic domain	99.3	100	97.1

^a The homology of hMuV F and batMuV F was compared to that of an MuV F consensus sequence which was derived from an alignment of the aa sequence of the F proteins of 26 MuV strains (GenBank accession numbers EU884413.1, FJ556896.1, AF467767.2, GU980052.1, AF338106, AY681495.1, AY508995, AB600843.1, JX287386.1, EU370206.3, JX287388.1, AY309060.1, AB600942.1, AF314561.1, AF314558.1, AB823535.1, JN012242.1, KF481689.1, AF280799.1, EU370207.1, JN635498.1, JX287385.1, JX287391.1, JX287390.1, JX287387.1, and JX287389.1), using the PRofile ALiGNeMent (PRALINE) software.

^b hMuV F designates the fusion protein of the clinical isolate used in this study.

^c The following SP sequences were used: consensus MuV F, MKAFVLVTCLGFAVFSSSICV; hMuV F,S.....; batMuV F, .RKT.AIG...ML.L.V.I.

was compared to that of a consensus sequence derived from 26 full-length sequences of human MuV F (Table 1). The conservation of the full-length F sequence as well as of different functional domains within the F protein of our clinical isolate, hMuV, was within the range of variation observed among the published sequences. Overall, the amino acid similarity of the fusion protein of batMuV to that of the MuV consensus sequence was very high: 94.6% for the full-length protein, 96.5% for F₁, and 86.5% for the F₂ subunit (Table 1). A striking difference was noticed in the first 20 amino acids of the F₂ subunit comprising the putative signal peptide. There, at the amino terminus of F₂, the amino acid homology between the MuV consensus sequence and batMuV was only 69% (Table 1). In the case of our clinical isolate, this predicted signal peptide differs only at position 5 from the consensus sequence (serine instead of leucine). Therefore, chimeric F proteins with an exchange of the first 20 (putative SP) or 102 (F₂ subunit) amino acids were generated (Fig. 5) and further analyzed in a fusion assay. In coexpression with hMuV or batMuV HN, F proteins containing the hMuV F₂ subunit or only the hMuV F SP induced the formation of huge syncytia, whereas syncytia medi-

FIG 5 Schematic illustration of MuV F chimera. SP, signal peptide; ED, ectodomain; TD, transmembrane domain; CT, cytoplasmic tail. The black arrowhead indicates the cleavage site.

ated by the F proteins containing the bat-derived F₂ subunit or signal peptide were much smaller (Fig. 6).

To determine if the hMuV F SP enables more efficient protein processing and transport along the secretory pathway than the batMuV F SP, the intracellular and surface expression of hMuV F, batMuV F, and the SP chimera was analyzed by IFA and flow cytometry. To exclude differences in the binding affinities of the antibodies to the human- or bat-derived F proteins, HA epitope-labeled proteins were generated and expressed in BHK-21 cells. The fluorescence observed in cells permeabilized by Triton X-100 (+TX) and nonpermeabilized cells (−TX) was similar for hMuV F-HA and hMuV F SP-HA (Fig. 7A), suggesting that there are no significant differences in the intracellular and surface expression levels of these proteins. In contrast, the fluorescence observed with the cells expressing the batMuV F-HA or batMuV F SP-HA appeared to be reduced both with respect to the fluorescence intensity and to the number of fluorescent cells. To quantify the surface expression, GFP- and MuV F-cotransfected cells were counted by IFA. The mean number of GFP-positive cells, given as a percentage of the total cell number, was similar between all samples, indicating similar transfection efficiencies (data not shown). Differences were obtained when the number of cells showing surface expression of the MuV F constructs was determined. Compared to cells with surface expression of hMuV F-HA (set as 100%), the respective values for hMuV F SP-HA, batMuV F-HA, and batMuV F SP-HA were 96, 67, and 62%, respectively (Fig. 7B). When the surface expression of the MuV F proteins was analyzed by flow cytometry, a similar result was obtained. The percentage of cells which express hMuV F-HA or hMuV F SP-HA on the surface was higher than that of the cells which express batMuV F-HA or batMuV F SP-HA (Fig. 7C). Further-

more, consistent with the findings obtained by IFA (Fig. 7A), a greater amount of cells with high fluorescence intensity was found for hMuV F-HA and hMuV F SP-HA (Fig. 7C). For batMuV F-HA and batMuV F SP-HA, most of the cells showed lower fluorescence intensities, and only a few cells with higher fluorescence intensity were detected.

DISCUSSION

The identification of genomic sequences of a bat virus related to MuV was a unique finding because it had revealed that a virus that was believed to have humans as its only host has substantial similarity to a counterpart in bats (9). Sequence analysis and serological assays suggested that hMuV and batMuV are conspecific and belong to one serogroup. The antigenic relatedness was confirmed in this study. Mono- or polyclonal antibodies directed against the F or HN proteins of different MuV strains efficiently interacted with the corresponding proteins of batMuV, as indicated by immunofluorescence microscopy of transfected cells. As the F and HN proteins of paramyxoviruses are major targets of neutralizing antibodies, it is conceivable that the protective immunity elicited by MuV infection or vaccination applies to batMuV. Although an infectious batMuV is not yet available, one can predict that this virus will not succeed in infecting humans that carry neutralizing antibodies against human MuV strains. On the other hand, it remains to be analyzed whether persons that lack neutralizing antibodies are susceptible to infection by batMuV. Furthermore, the possibility should be considered that there are batMuV variants which are less serologically related to human strains. If such viruses are able to infect humans, they might evade the immune systems of people with antibodies against hMuV.

FIG 6 Coexpression of MuV F chimera and MuV HN proteins. BHK-21 cells were grown on coverslips and transfected for the coexpression of hMuVF₂, batMuVF₂, hMuV F SP, or batMuV F SP and hMuV HN/batMuV HN. At 12 h p.t., cells were fixed with methanol-acetone (1:1) and incubated in May Grünwald and Giemsa staining solution. Black arrows indicate syncytia. The scale bar indicates 50 μ m.

In addition to the serological similarity, the results presented in this study also demonstrate a functional relatedness between the glycoproteins of hMuV and batMuV. Like the human counterpart, the HN protein of batMuV has a sialic acid binding activity that is evident in hemadsorption activity. In the case of MuV, adaptation to different hosts (humans and bats) did not involve a major change in receptor binding activity. This is different from coronaviruses. Although the receptor for the vast majority of severe acute respiratory syndrome (SARS)-like bat viruses is not known, the S proteins of these viruses appear to interact with a receptor different from that of human ACE2 (35–37). The same may apply to influenza viruses. Genomic sequences detected in bats from Central America were used to generate a recombinant bat influenza virus from cDNA (38, 39). These attempts were successful only when the glycoproteins of the bat virus were replaced by those of an avian influenza virus. The receptor for bat influenza viruses is not known; however, the available evidence indicates that the hemagglutinins of these viruses are not able to use sialic acid as a receptor determinant (40–44).

The HN protein of batMuV resembled the corresponding protein of hMuV in its second biological activity, the ability to release sialic acids from sialoglycoconjugates. The presence of neuraminidase activity was more difficult to demonstrate, because the values above background levels were obtained only when the transfection reagent used for the initial experiments was replaced by another one. The neuraminidase activity of the surface-expressed batMuV HN was lower than that of the hMuV counterpart. This finding can be explained by the lower expression level of the batMuV HN protein, suggesting that the specific enzyme activities of the hMuV and batMuV HN proteins are similar.

Similar to most paramyxoviruses, the fusion activity of batMuV requires the cooperation of the F and the HN protein. The functional relatedness between hMuV and batMuV is convincingly demonstrated by the finding that each of the two glycoproteins of batMuV can cooperate with the respective partner protein of hMuV to induce syncytium formation. Among paramyxoviruses, the fusion protein usually requires the HN protein of viruses from the same species to induce membrane fusion. An exception is simian virus 5 (SV5), which can cooperate with HN proteins of other paramyxoviruses (45). However, the F protein of SV5 is unusual because it has fusion activity even in the absence of HN protein. Although there are substantial differences in the small hydrophobic (SH) protein between batMuV and MuV with a homology value of 61% with respect to a consensus sequence for MuV strains, the similarity of the other viral proteins is rather high, with homology values of about 90%. This similarity is sufficient to allow cooperation of the glycoproteins in the induction of syncytium formation. However, there also are differences between hMuV and batMuV in their fusion activity. Syncytia induced by the bat virus glycoproteins were substantially smaller than those observed after coexpression of the glycoproteins of hMuV. A similar difference has been demonstrated recently when the syncytium formation by a putative African henipavirus was compared to that induced by the Nipah virus glycoproteins (33, 46, 47). Cooperation of the F and G proteins of the latter virus results in large syncytia in all mammalian cells. In contrast, syncytium formation induced by the glycoproteins of the African henipavirus is restricted to chiropteran cells and not observed in cells of other mammalian species (33, 46, 47). This differential fusion activity has been traced back to the surface transport of the G protein, which was inefficient in all cells, but the inefficiency was less pronounced in chiropteran cells (28). The fusion activity of the batMuV glycoproteins differs from that of the African henipavirus, as it is not restricted to chiropteran cells. Similar to the African henipavirus, the intracellular and surface expression of the attachment glycoprotein of batMuV was lower than that of its hMuV counterpart. The same is true for the chimeric MuV F proteins containing the F₂ or the signal peptide of the fusion protein of the human virus. As shown by analysis of the chimeric proteins, the signal peptide is the determinant which downregulates the fusion activity of the F protein of batMuV. The finding that MuV F proteins which contain the hMuV F SP show higher expression levels than those of F proteins containing the batMuV F SP suggests that the MuV F SP affects the efficiency of protein processing and transport along the secretory pathway. In this context, it is interesting that several bat viruses show restrictions in the entry strategies. As mentioned above, SARS-like bat coronaviruses and bat influenza viruses are restricted at the level of interaction with cellular receptors (35), and the African henipavirus and batMuV are restricted in their fusion activity (33).

FIG 7 Intracellular and surface expression of MuV F proteins and F chimera. (A) BHK-21 cells were transfected for the expression of hMuV F-HA, batMuV F-HA, and the HA-labeled SP chimera (hMuV F SP-HA and batMuV F SP-HA). Nonpermeabilized (–TX) cells were incubated with anti-HA rabbit antibodies. Cells next were permeabilized (+TX) and incubated with anti-HA mouse antibodies, followed by incubation with Alexa Fluor 488 anti-rabbit (green; surface expression) and Alexa Fluor 568 anti-mouse (red; intracellular expression) antibodies. The scale bar indicates 100 μ m. (B) Normalized surface expression of MuV F proteins and chimera. The surface expression was normalized to the surface expression of hMuV F-HA, which was set as 100%. The normalized surface expression is given as a percentage of positive cells. (C) Histogram plots of hMuV F, hMuV F SP, batMuV F, and batMuV F SP surface expression in BHK-21 cells. The gray curve represents the histogram plot of pCG1-transfected cells. The gate setting is indicated by the black line.

Therefore, replication of the respective bat viruses may not be characterized by production of a large number of progeny virions but rather by a persistent type of infection characterized by cell-to-cell spread. The difficulty in isolating these viruses in an infectious form is consistent with this idea. To get more knowledge about the replication of these bat viruses, it is, however, indispensable to isolate infectious viruses from the respective bats in the future.

ACKNOWLEDGMENTS

N.K. was funded by a fellowship of the Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology, and Translational Medicine (HGNI). This work was supported by grants to G.H. from DFG (HE 1168/14-1 and SFB621 TP B7) and from Bundesministerium für Bildung und Forschung (Ecology and Pathogenesis of SARS, project code 01KI1005B, and FluResearchNet, project code 01KI1006D).

We are grateful to Roberto Cattaneo for providing expression plasmids. We also thank Silke Rautenschlein and Christine Hase from the Clinic for Poultry, University of Veterinary Medicine Hannover, for providing us with chicken erythrocytes.

REFERENCES

- Afzal MA, Buchanan J, Heath AB, Minor PD. 1997. Clustering of mumps virus isolates by SH gene sequence only partially reflects geographical origin. *Arch Virol* 142:227–238. <http://dx.doi.org/10.1007/s007050050073>.
- Orvell C, Kalantari M, Johansson B. 1997. Characterization of five conserved genotypes of the mumps virus small hydrophobic (SH) protein gene. *J Gen Virol* 78(Part 1):91–95.
- Tecle T, Johansson B, Jecic A, Forsgren M, Orvell C. 1998. Characterization of three co-circulating genotypes of the small hydrophobic protein gene of mumps virus. *J Gen Virol* 79(Part 12):2929–2937.
- Yeo RP, Afzal MA, Forsey T, Rima BK. 1993. Identification of a new mumps virus lineage by nucleotide sequence analysis of the SH gene of ten different strains. *Arch Virol* 128:371–377. <http://dx.doi.org/10.1007/BF01309447>.
- WHO. 2012. Mumps virus nomenclature update: 2012. *Wkly Epidemiol Rec* 87(22):217–224.
- CDC. 2012. Mumps. In Atkinson WM, Wolfe C, Hamborsky J (ed), *Epidemiology and prevention of vaccine-preventable diseases*, 12th ed. Centers for Disease Control and Prevention, Atlanta, GA.
- Beard CM, Benson RC, Jr, Kelalis PP, Elveback LR, Kurland LT. 1977. The incidence and outcome of mumps orchitis in Rochester, Minnesota, 1935 to 1974. *Mayo Clin Proc* 52:3–7.
- Russell RR, Donald JC. 1958. The neurological complications of mumps. *Br Med J* 2:27–30. <http://dx.doi.org/10.1136/bmj.2.5087.27>.
- Drexler JF, Corman VM, Muller MA, Maganga GD, Vallo P, Binger T, Gloza-Rausch F, Rasche A, Yordanov S, Seebens A, Oppong S, Adu Sarkodie Y, Pongombo C, Lukashev AN, Schmidt-Chanasit J, Stocker A, Carneiro AJ, Erbar S, Maisner A, Fronhoffs F, Buettner R, Kalko EK, Kruppa T, Franke CR, Kallies R, Yandoko ER, Herrler G, Reusken C, Hassanin A, Kruger DH, Matthee S, Ulrich RG, Leroy EM, Drosten C. 2012. Bats host major mammalian paramyxoviruses. *Nat Commun* 3:796. <http://dx.doi.org/10.1038/ncomms1796>.
- Samal SK. 2011. *The biology of paramyxoviruses*. Caister Academic Press, Norfolk, United Kingdom.
- Chu LW, Morgan HR. 1950. Studies of the hemolysis of red blood cells by mumps virus. II. The relationships of hemagglutination, virus elution, and hemolysis. *J Exp Med* 91:403–416.
- Morgan HR, Enders JF, Wagley PF. 1948. A hemolysin associated with the mumps virus. *J Exp Med* 88:503–514. <http://dx.doi.org/10.1084/jem.88.5.503>.
- Burnet FM, McEwin J. 1945. Agglutination of pigeon erythrocytes in influenza virus A in the 0 phase. *Aust J Exp Biol Med Sci* 23:189–192.
- Beveridge WI, Lind PE. 1946. Mumps; virus haemagglutination and serological reactions. *Aust J Exp Biol Med Sci* 24:127–132. <http://dx.doi.org/10.1038/icb.1946.20>.
- Challut F, Chardonnet Y, Favier G, Sohler R. 1956. Hemagglutinating

- capacity of nine strains of mumps virus towards erythrocytes of various species. *Ann Inst Pasteur (Paris)* 91:137–146.
16. Hosaka Y, Kuroda K, Ikeura A, Iwamoto T, Suzuki Y. 1998. Binding of influenza and paramyxoviruses to group B Streptococcus with the terminal sialyl-galactose linkage. *J Electron Microscop (Tokyo)* 47:169–174. <http://dx.doi.org/10.1093/oxfordjournals.jmicro.a023574>.
 17. Brostrom MA, Bruening G, Bankowski RA. 1971. Comparison of neuraminidases of paramyxoviruses with immunologically dissimilar hemagglutinins. *Virology* 46:856–865. [http://dx.doi.org/10.1016/0042-6822\(71\)90086-9](http://dx.doi.org/10.1016/0042-6822(71)90086-9).
 18. Leprat R, Aymard M. 1979. Selective inactivation of hemagglutinin and neuraminidase on mumps virus. *Arch Virol* 61:273–281. <http://dx.doi.org/10.1007/BF01315013>.
 19. Santos-Lopez G, Scior T, del Borraz-Arguello MT, Vallejo-Ruiz V, Herrera-Camacho I, Tapia-Ramirez J, Reyes-Leyva J. 2009. Structure-function analysis of two variants of mumps virus hemagglutinin-neuraminidase protein. *Braz J Infect Dis* 13:24–34. <http://dx.doi.org/10.1590/S1413-86702009000100007>.
 20. Wolinsky JS, Stroop WG. 1978. Virulence and persistence of three prototype strains of mumps virus in newborn hamsters. *Arch Virol* 57:355–359. <http://dx.doi.org/10.1007/BF01320075>.
 21. McCarthy M, Johnson RT. 1980. A comparison of the structural polypeptides of five strains of mumps virus. *J Gen Virol* 46:15–27. <http://dx.doi.org/10.1099/0022-1317-46-1-15>.
 22. Merz DC, Wolinsky JS. 1981. Biochemical features of mumps virus neuraminidases and their relationship with pathogenicity. *Virology* 114:218–227. [http://dx.doi.org/10.1016/0042-6822\(81\)90267-1](http://dx.doi.org/10.1016/0042-6822(81)90267-1).
 23. Waxham MN, Server AC, Goodman HM, Wolinsky JS. 1987. Cloning and sequencing of the mumps virus fusion protein gene. *Virology* 159:381–388. [http://dx.doi.org/10.1016/0042-6822\(87\)90477-6](http://dx.doi.org/10.1016/0042-6822(87)90477-6).
 24. Rubin S, Vandermeulen C. 2011. Mumps virus. In Samal SK (ed), *The biology of paramyxoviruses*, p 5–36. Caister Academic Press, Norfolk, United Kingdom.
 25. Simossis VA, Heringa J. 2003. The PRALINE online server: optimising progressive multiple alignment on the web. *Comput Biol Chem* 27:511–519. <http://dx.doi.org/10.1016/j.compbiolchem.2003.09.002>.
 26. Simossis VA, Heringa J. 2005. PRALINE: a multiple sequence alignment toolbox that integrates homology-extended and secondary structure information. *Nucleic Acids Res* 33:W289–W294. <http://dx.doi.org/10.1093/nar/gki390>.
 27. Hoffmann M, Muller MA, Drexler JF, Glende J, Erdt M, Gutzkow T, Losenmann C, Binger T, Deng H, Schwegmann-Wessels C, Esser KH, Drosten C, Herrler G. 2013. Differential sensitivity of bat cells to infection by enveloped RNA viruses: coronaviruses, paramyxoviruses, filoviruses, and influenza viruses. *PLoS One* 8:e72942. <http://dx.doi.org/10.1371/journal.pone.0072942>.
 28. Kruger N, Hoffmann M, Drexler JF, Muller MA, Corman VM, Drosten C, Herrler G. 2014. Attachment protein G of an African bat henipavirus is differentially restricted in chiropteran and nonchiropteran cells. *J Virol* 88:11973–11980. <http://dx.doi.org/10.1128/JVI.01561-14>.
 29. Biesold SE, Ritz D, Gloza-Rausch F, Wollny R, Drexler JF, Corman VM, Kalko EK, Oppong S, Drosten C, Muller MA. 2011. Type I interferon reaction to viral infection in interferon-competent, immortalized cell lines from the African fruit bat *Eidolon helvum*. *PLoS One* 6:e28131. <http://dx.doi.org/10.1371/journal.pone.0028131>.
 30. Knoppova M, Phensajai M, Vesely M, Zemanova M, Nesvera J, Patek M. 2007. Plasmid vectors for testing in vivo promoter activities in *Corynebacterium glutamicum* and *Rhodococcus erythropolis*. *Curr Microbiol* 55:234–239. <http://dx.doi.org/10.1007/s00284-007-0106-1>.
 31. Orvell C, Alsheikhly AR, Kalantari M, Johansson B. 1997. Characterization of genotype-specific epitopes of the HN protein of mumps virus. *J Gen Virol* 78(Part 12):3187–3193.
 32. Tisoncik JR, Guo Y, Cordero KS, Yu J, Wang J, Cao Y, Rong L. 2011. Identification of critical residues of influenza neuraminidase in viral particle release. *Virol J* 8:14. <http://dx.doi.org/10.1186/1743-422X-8-14>.
 33. Kruger N, Hoffmann M, Weis M, Drexler JF, Muller MA, Winter C, Corman VM, Gutzkow T, Drosten C, Maisner A, Herrler G. 2013. Surface glycoproteins of an African henipavirus induce syncytium formation in a cell line derived from an African fruit bat, *Hypsignathus monstrosus*. *J Virol* 87:13889–13891. <http://dx.doi.org/10.1128/JVI.02458-13>.
 34. Orvell C. 1984. The reactions of monoclonal antibodies with structural proteins of mumps virus. *J Immunol* 132:2622–2629.
 35. Ren W, Qu X, Li W, Han Z, Yu M, Zhou P, Zhang SY, Wang LF, Deng H, Shi Z. 2008. Difference in receptor usage between severe acute respiratory syndrome (SARS) coronavirus and SARS-like coronavirus of bat origin. *J Virol* 82:1899–1907. <http://dx.doi.org/10.1128/JVI.01085-07>.
 36. Hou Y, Peng C, Yu M, Li Y, Han Z, Li F, Wang LF, Shi Z. 2010. Angiotensin-converting enzyme 2 (ACE2) proteins of different bat species confer variable susceptibility to SARS-CoV entry. *Arch Virol* 155:1563–1569. <http://dx.doi.org/10.1007/s00705-010-0729-6>.
 37. Becker MM, Graham RL, Donaldson EF, Rockx B, Sims AC, Sheahan T, Pickles RJ, Corti D, Johnston RE, Baric RS, Denison MR. 2008. Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *Proc Natl Acad Sci U S A* 105:19944–19949. <http://dx.doi.org/10.1073/pnas.0808116105>.
 38. Juozapaitis M, Aguiar Moreira E, Mena I, Giese S, Riegger D, Pohlmann A, Hoper D, Zimmer G, Beer M, Garcia-Sastre A, Schwemmle M. 2014. An infectious bat-derived chimeric influenza virus harbouring the entry machinery of an influenza A virus. *Nat Commun* 5:4448. <http://dx.doi.org/10.1038/ncomms5448>.
 39. Zhou B, Ma J, Liu Q, Bawa B, Wang W, Shabman RS, Duff M, Lee J, Lang Y, Cao N, Nagy A, Lin X, Stockwell TB, Richt JA, Wentworth DE, Ma W. 2014. Characterization of uncultivable bat influenza virus using a replicative synthetic virus. *PLoS Pathog* 10:e1004420. <http://dx.doi.org/10.1371/journal.ppat.1004420>.
 40. Tong S, Li Y, Rivallier P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO. 2012. A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci U S A* 109:4269–4274. <http://dx.doi.org/10.1073/pnas.1116200109>.
 41. Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO. 2013. New world bats harbor diverse influenza A viruses. *PLoS Pathog* 9:e1003657. <http://dx.doi.org/10.1371/journal.ppat.1003657>.
 42. Sun X, Shi Y, Lu X, He J, Gao F, Yan J, Qi J, Gao GF. 2013. Bat-derived influenza hemagglutinin H17 does not bind canonical avian or human receptors and most likely uses a unique entry mechanism. *Cell Rep* 3:769–778. <http://dx.doi.org/10.1016/j.celrep.2013.01.025>.
 43. Wu Y, Wu Y, Tefsen B, Shi Y, Gao GF. 2014. Bat-derived influenza-like viruses H17N10 and H18N11. *Trends Microbiol* 22:183–191. <http://dx.doi.org/10.1016/j.tim.2014.01.010>.
 44. Zhu X, Yu W, McBride R, Li Y, Chen LM, Donis RO, Tong S, Paulson JC, Wilson IA. 2013. Hemagglutinin homologue from H17N10 bat influenza virus exhibits divergent receptor-binding and pH-dependent fusion activities. *Proc Natl Acad Sci U S A* 110:1458–1463. <http://dx.doi.org/10.1073/pnas.1218509110>.
 45. Bagai S, Lamb RA. 1995. Quantitative measurement of paramyxovirus fusion: differences in requirements of glycoproteins between simian virus 5 and human parainfluenza virus 3 or Newcastle disease virus. *J Virol* 69:6712–6719.
 46. Lawrence P, Escudero Perez B, Drexler JF, Corman VM, Muller MA, Drosten C, Volchkov V. 2014. Surface glycoproteins of the recently identified African henipavirus promote viral entry and cell fusion in a range of human, simian and bat cell lines. *Virus Res* 181:77–80. <http://dx.doi.org/10.1016/j.virusres.2014.01.003>.
 47. Weis M, Behner L, Hoffmann M, Kruger N, Herrler G, Drosten C, Drexler JF, Dietzel E, Maisner A. 2014. Characterization of African bat henipavirus GH-M74a glycoproteins. *J Gen Virol* 95:539–548. <http://dx.doi.org/10.1099/vir.0.060632-0>.