JOURNAL OF VIROLOGY, Apr. 2005, p. 4425–4433 Vol. 79, No. 7
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.7.4425–4433.2005
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RNA Polymerase I-Driven Minigenome System for Ebola Viruses

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Received 26 August 2004/Accepted 12 November 2004

In general, Ebola viruses are well known for their ability to cause severe hemorrhagic fever in both human and nonhuman primates. However, despite substantial sequence homology to other members of the family *Filoviridae*, *Reston ebolavirus* displays reduced pathogenicity for nonhuman primates and has never been demonstrated to cause clinical disease in humans, despite its ability to cause infection. In order to develop a tool to explore potential roles for transcription and replication in the reduced pathogenicity of *Reston ebolavirus*, we developed an RNA polymerase I (Pol I)-driven minigenome system. Here we demonstrate successful *Reston ebolavirus* minigenome rescue, including encapsidation, transcription, and replication, as well as the packaging of minigenome transcripts into progeny particles. The Pol I-driven *Reston ebolavirus* minigenome system provides a higher signal intensity with less background (higher signal-to-noise ratio) than a comparable T7-driven *Reston ebolavirus* minigenome system which was developed simultaneously. Successful *Reston ebolavirus* minigenome rescue was also achieved by the use of helper plasmids derived from the closely related *Zaire ebolavirus* or the more distantly related *Lake Victoria marburgvirus*. The use of heterologous helper plasmids in the *Reston ebolavirus* minigenome system yielded levels of reporter expression which far exceeded the level produced by the homologous helper plasmids. This comparison between minigenomes and helper plasmids from different filovirus species and genera indicates that inherent differences in the transcription and/or replication capacities of the ribonucleoprotein complexes of pathogenic and apathogenic filoviruses may exist, as these observations were confirmed in a *Lake Victoria marburgvirus* minigenome system.

The family *Filoviridae* within the order *Mononegavirales* contains two genera, *Marburgvirus* (MARV) and *Ebolavirus* (EBOV). The genus *Ebolavirus* is further subdivided into four distinct species, *Côte d'Ivoire ebolavirus*, *Reston ebolavirus* (REBOV), *Sudan ebolavirus*, and *Zaire ebolavirus* (ZEBOV) (4). In terms of biohazards, filoviruses, which cause a lethal hemorrhagic fever in both human and nonhuman primates, are classified as biosafety level (BSL) 4 agents based on their high mortality rates, person-to-person transmission, and potential aerosol infectivities and the absence of vaccines or chemotherapeutic agents for these viruses (21).

REBOV emerged in 1989–1990 as the causative agent of an epizootic among groups of cynomolgus macaques (*Macaca fascicularis*) imported from the Philippines into the United States (18). Subsequently, at least two more introductions of REBOV have occurred in the United States and Italy (32, 37). Despite its pathogenicity for nonhuman primates (8), REBOV has never been associated with any notable disease in humans. However, serological investigations have documented at least eight seroconversions among exposed animal handlers (1, 2, 23, 24), suggesting that REBOV infections in humans lead to either asymptomatic or subclinical courses of disease. Furthermore, there are some data suggesting that REBOV also displays a lower pathogenicity for nonhuman primates (8). These properties make REBOV an interesting species for the study of filovirus pathogenicity, particularly in comparison to the most virulent species, ZEBOV. Among other strategies, reverse genetic approaches provide powerful tools with which to perform these types of analyses.

Filoviruses contain single-stranded, negative-sense, nonsegmented RNA genomes of approximately 19 kb (19.1 kb for MARV and 18.9 kb for EBOV). The gene order is as follows: 3′ leader–nucleoprotein (NP)–virion protein 35 (VP35)–VP40–glycoprotein (GP)–VP30–VP24–RNA-dependent RNA polymerase (L)–5′ trailer. Transcription and translation result in the synthesis of seven structural proteins with presumed identical functions for all of the different filoviruses. In addition, a single nonstructural secreted glycoprotein is expressed by all EBOV, but not by MARV (6, 7, 33). Four of the virion structural proteins, NP, VP30 (transcription factor), VP35 (polymerase cofactor), and L (RNA-dependent RNA polymerase), are associated with the viral genomic RNA to form the ribonucleoprotein (RNP) complex. These proteins have been shown to be necessary and sufficient for EBOV transcription and replication (3, 6, 25, 26). As a result, it is possible to supply these components along with a minigenome, in which the virus genes are replaced by an assayable reporter, in order to model the processes of encapsidation, transcription, replication, and packaging. This is possible because all of the minimal essential signals for these processes are conserved within the viral 3′ and

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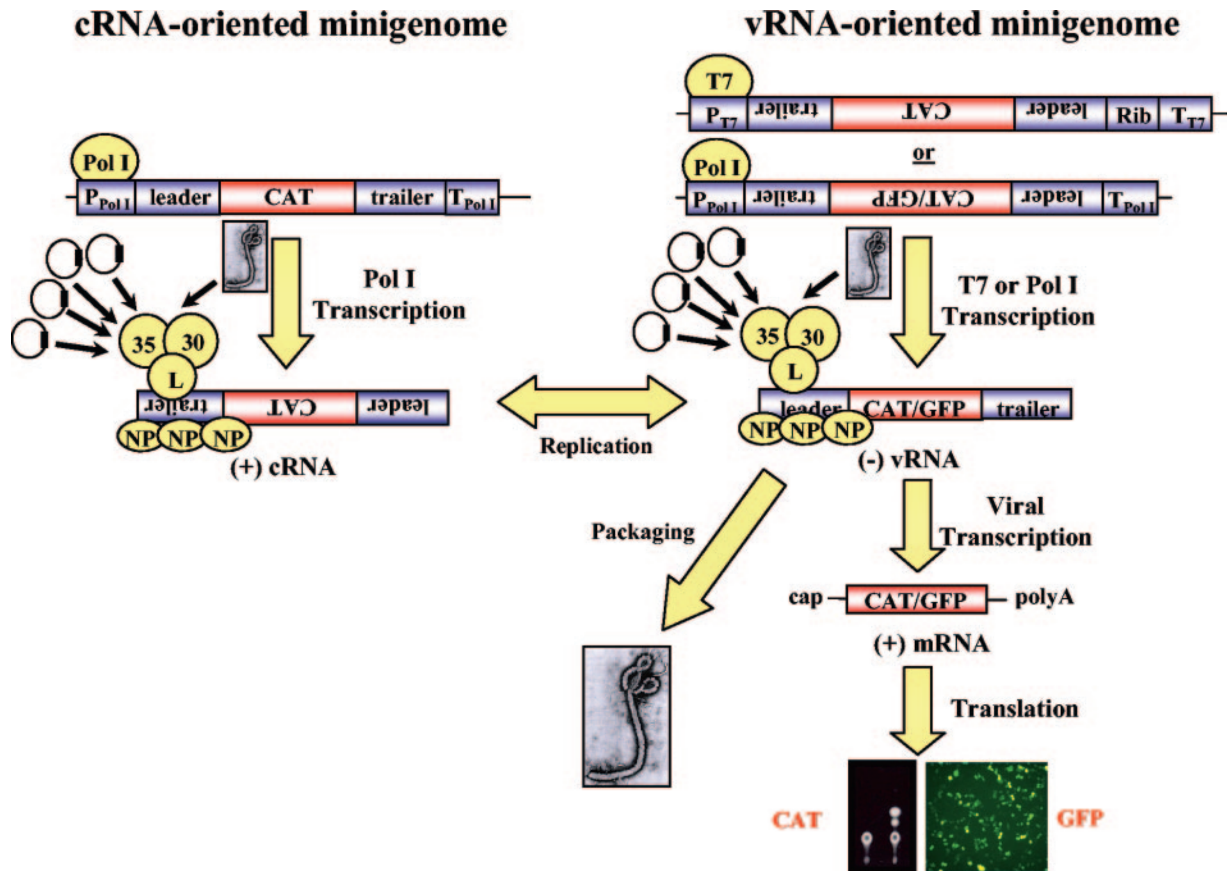

FIG. 1. Schematic representation of reporter gene expression from vRNA- and cRNA-oriented T7- and Pol I-driven minigenomes. Reporter minigenomes were composed of a reporter gene, either CAT or GFP, flanked by the 3′ (leader) and 5′ (trailer) noncoding regions of REBOV. These transcription cassettes were then cloned between the transcriptional start and stop signals for either the bacteriophage T7 polymerase or human RNA Pol I. In the case of the T7 minigenome, an HDV ribozyme sequence was cloned immediately adjacent to the viral leader to ensure a correct end to the transcript cleavage. Transcription of the transcription cassette by Pol I or T7 from the appropriate promoter results in the production of either a vRNA-like (vRNA-oriented construct) or a cRNA-like (cRNA-oriented construct) molecule. The vRNA-like transcript can interact with the viral RNA complex proteins, which are supplied in *trans* by either helper plasmid cotransfection or REBOV infection, to mediate further transcription of a reporter mRNA molecule. This transcript can then be translated to produce the active reporter protein. After transcription of the cRNA-like molecule, replication to a vRNA-like molecule must first occur before mRNA transcription and protein translation can occur. L, RNA-dependent RNA polymerase; NP, nucleoprotein; 30 and 35, VP30 and VP35, respectively.

5′ noncoding regions, which are incorporated as part of the minigenome (25, 26). Thus, minigenomes provide powerful tools to study these processes and can eliminate biosafety concerns associated with the use of infectious virus.

MATERIALS AND METHODS

Cells and virus. 293T (human embryonic kidney) and Vero E6 (ATCC CRL 1586; African green monkey [*Cercopithecus aethiops*] kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Burlington, Ontario) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml. REBOV (strain Pennsylvania) and ZEBOV (strain Mayinga) were kindly provided by the Special Pathogens Branch of the Centers for Disease Control and Prevention (Atlanta, Ga.). All experiments with infectious virus were preformed in the BSL-4 laboratory at the National Microbiology Laboratory, Public Health Agency of Canada.

Virus propagation and RNA isolation. For virus propagation, Vero E6 cells were infected at a multiplicity of infection (MOI) of 0.01 PFU. After an adsorption period of 1 h, the cells were incubated in DMEM (Invitrogen) containing 2% FCS (Invitrogen) for 7 (ZEBOV) or 14 (REBOV) days. RNA extraction was performed according to the manufacturer's instructions by use of an RNeasy kit (QIAGEN, Mississauga, Ontario) as previously described (15).

Plasmid construction. (i) Pol I minigenome construction. In order to generate a Pol I-driven REBOV minigenome construct, we cloned the viral 3′ (leader) and 5′ (trailer) noncoding regions flanking either the chloramphenicol acetyltransferase (CAT) or green fluorescent protein (GFP) reporter gene. This cassette was produced by ligation of a BbsI-digested leader amplicon to a CAT or GFP amplicon bearing compatible overhangs generated by BsaI (CAT) or BsmBI (GFP) cleavage, respectively. The resulting fragment was PCR amplified and cloned into the human Pol I promoter- and terminator-containing vector pRF240 (11). This construct was further cleaved with BbsI, and the trailer was inserted, again by the use of overhangs generated by BbsI cleavage. The resulting construct contained either a CAT or GFP minigenome cassette in the viral RNA (vRNA) orientation and consisted of the following elements, in order, as shown in Fig. 1: Pol I promoter-trailer-reporter-leader-Pol I terminator. In order to construct a cRNA-transcribing Pol I-driven minigenome, we amplified the entire reporter cassette (consisting of the CAT gene flanked by the leader and trailer regions) from the corresponding vRNA construct. This fragment was then inserted into pRF240 in a directional fashion by the use of overhangs generated with BbsI. The resulting construct contained the CAT minigenome cassette in the cRNA orientation and consisted of the following elements, in order, as shown in Fig. 1: Pol I promoter-leader-reporter-trailer-Pol I terminator.

(ii) vRNA-oriented T7 minigenome construction. The generation of the REBOV vRNA-oriented T7-driven minigenome construct was based on similar systems established for ZEBOV and MARV, which contain an additional non-

coded G residue immediately upstream of the leader sequence (25, 26). The plasmid was produced by subcloning the reporter cassette from a vRNA-oriented Pol I-driven CAT construct into a T7 promoter-containing pBluescript-based vector (Stratagene, La Jolla, Calif.), which also contained the hepatitis delta virus (HDV) ribozyme and T7 terminator sequences. These elements were inserted into pBluescript by the use of EagI and SacI after PCR amplification from a T7-driven vesicular stomatitis virus genome-containing plasmid (kindly provided by J. Rose, Yale University, New Haven, Conn.). The CAT reporter cassette (consisting of the CAT gene flanked by the leader and trailer regions), amplified from the vRNA-oriented Pol I minigenome by the use of compatible ends produced by BbsI and KpnI cleavage, was then inserted into this construct. The final construct consisted of the following elements, as shown in Fig. 1: T7 promoter-G-trailer-reporter-leader-HDV ribozyme-T7 terminator. Additional constructs which contained zero, two, or three upstream G residues were produced by site-directed mutagenesis, but this was found to have no notable effect on reporter activity (data not shown), and therefore all experiments were preformed with the one-G-containing construct.

(iii) Expression plasmids. The open reading frames (ORFs) for the NP, VP35, VP30, and L genes of REBOV were generated by reverse transcription-PCR (RT-PCR) methodology using sequence-specific primers and were cloned into the vector pCAGGS, which mediates expression of the foreign gene under the control of a chicken β-actin-derived promoter (29). The entire ORFs for NP and VP35 were cloned by the use of EcoRI, while the VP30 ORF was cloned by the use of EcoRI and XhoI. In the case of the L gene, the construct was cloned in two steps by use of the vector's EcoRI and NheI sites. Since digestion of the insert with EcoRI was not possible due to the presence of multiple cleavage sites, an EcoRI-compatible overhang was generated by the use of BsaI. An NheI site in the L ORF was inserted via a silent mutation (A→G) at nucleotide 14834 to facilitate subsequent cloning steps. The remainder of the construct was inserted by use of this NheI site as well as the vector's BglII site. After sequence confirmation, the expression of NP, VP35, and VP30 was confirmed by immunoblot analysis of cell lysates and by immunofluorescence analysis of transfected cells with polyclonal sera raised against each of the proteins (A.Groseth, unpublished data). In addition, the proper functioning of all four protein products was confirmed based on their ability to mediate the rescue of virus from the ZEBOV infectious clone system (35).

Rescue of REBOV minigenomes. (i) Plasmid-driven system. 293T cells were seeded into six-well or 10-cm-diameter tissue culture plates and then transfected with various minigenome constructs, with or without helper plasmids encoding the RNP complex proteins, by the use of TransIT-LT1 (Mirus, Madison, Wis.). In the case of the T7-driven minigenome system, 1 μg of a T7 polymerase-encoding pCAGGS construct was also supplied (kindly provided by Y. Kawaoka, University of Wisconsin, Madison). Helper plasmids were supplied in various amounts to determine the optimal amount for each vector, with these amounts being used for all subsequent experiments. For each microgram of DNA to be transfected, 2 μl of transfection reagent was used according to the manufacturer's directions. OptiMEM serum-free medium (Invitrogen) was used for all transfections. Transfected samples were incubated for 48 h prior to CAT assays in the case of helper plasmid-driven experiments.

(ii) Helper virus-driven system. Transfected cells were superinfected at 24 h posttransfection of the minigenome with REBOV at an MOI of 0.1 to 0.01 PFU/cell. Briefly, 2 ml of diluted virus in DMEM without FCS and antibiotics was incubated with minigenome-transfected cells for 1 h in 10-cm-diameter cell culture plates (37°C, 5% CO_2). After incubation, the virus was removed and 6 ml of fresh DMEM (with 2% FCS and antibiotics) was added. Cultures were incubated for a further 24 to 72 h at 37°C with 5% CO_2 prior to harvesting for CAT assays.

CAT assays. CAT activity was assayed by use of the commercially available FastCAT system (Molecular Probes Inc., Eugene, Oreg.) according to the manufacturer's instructions. For all cotransfection experiments, 5% of the cell lysate was used, while for experiments in which RNP complex proteins were supplied by virus infection, 20% of the cell lysate was used. Reactions were incubated for either 2 or 18 h, as indicated. The results were documented by photography and/or evaluated with ImageQuant software (Amersham Biosciences Inc., Baie d'Urfé, Quebéc) for a Typhoon phosphorimager (Amersham Biosciences Inc.).

UV microscopy. Cells transfected with GFP-containing reporter minigenomes were assessed for GFP expression by use of an Axiovert 200 M microscope (Carl Zeiss Canada Ltd., Toronto, Ontario). Documentation was performed with an AxioCam HRm color video camera and the AxioVision imaging software package (Carl Zeiss Canada Ltd.).

Passaging of recombinant REBOV. 293T cells were transfected with 0.5 μg of the vRNA-oriented Pol I-driven CAT minigenome, as well as RNP complex protein-encoding plasmids, as described above. Transfected cell cultures were infected 24 h later with REBOV at an MOI of 0.01 PFU/cell. The cells were assayed for CAT activity at 72 h postinfection, and the corresponding supernatants were passaged on fresh cell monolayers. Debris was removed by low-speed centrifugation, and Vero E6 cells (approximately 10^6) were infected with 2 ml of undiluted supernatant (passage 1). After 1 h of incubation (37°C, 5% CO_2), the inoculum was replaced with fresh DMEM (with 2% FCS and antibiotics) and the cells were incubated for a further 72 h at 37°C with 5% CO_2. This process was repeated twice (passages 2 and 3). For determination of the CAT activity, 5% of the total cell lysate was incubated for 2 h in the presence of chloramphenicol and acetyl coenzyme A.

RESULTS

Analysis and optimization of Pol I-driven minigenome transcription. The generation of the different Pol I- and T7-driven reporter minigenome constructs is outlined in Materials and Methods and shown schematically in Fig. 1. In order to evaluate the function of the minigenome constructs prior to the generation of the appropriate expression plasmids required to drive transcription and replication entirely from plasmid DNA, we initially used a helper virus infection with REBOV to mediate reporter gene expression. Different amounts of the Pol I-driven CAT minigenome plasmid were transfected into 293T cells, followed 24 h later by infection with REBOV at an MOI of 0, 0.1, or 0.01. Samples were harvested at different time points postinfection and analyzed for CAT activity. Figure 2A shows the data obtained at 72 h postinfection, as this was determined to be the time of maximum reporter accumulation (data not shown). At this time point, the highest signal-to noise ratios were obtained with 250 ng of transfected minigenome and an MOI of 0.01. This decrease in reporter rescue observed with a higher MOI may be related to virus-induced changes in host cell functions that affect protein translation. Nevertheless, the helper virus infection clearly demonstrated that the minigenome constructs were recognized and subsequently transcribed by the viral polymerase complex.

In order to improve the system and to circumvent the need to use infectious REBOV and thus to perform the work under BSL-4 conditions, we needed to establish a set of conditions which would allow reporter transcription in a plasmid-driven system. For this system, we began by transfecting various amounts of the Pol I-driven vRNA-oriented CAT minigenome construct in the presence or absence of the necessary helper plasmids (encoding NP, VP35, VP30, and L). The amounts of these plasmids supplied were based on the optimal values previously determined for the T7-driven ZEBOV minigenome and infectious clone systems (26, 27) and were as follows: NP, 1.0 μg; VP35, 0.5 μg; VP30, 0.3 μg; L, 1.0 μg. These values were subsequently confirmed to be optimal for the transcription of REBOV minigenomes as well (Fig. 3). Based on these input minigenome titration experiments, it was found that the transfection of 250 ng of vRNA-oriented Pol I-driven CAT minigenome was sufficient to produce a CAT activity nearly equal to that obtained with larger amounts of reporter (500 ng of transfected minigenome) in the presence of the required helper plasmids (Fig. 2B). Importantly, there was no detectable background reporter activity in the absence of helper plasmids when this amount of transfected minigenome was used (Fig. 2B). The background activity observed in the presence of larger amounts of reporter minigenome likely results from the recognition of cryptic promoter elements contained within the minigenome plasmid by host polymerases (9, 13).

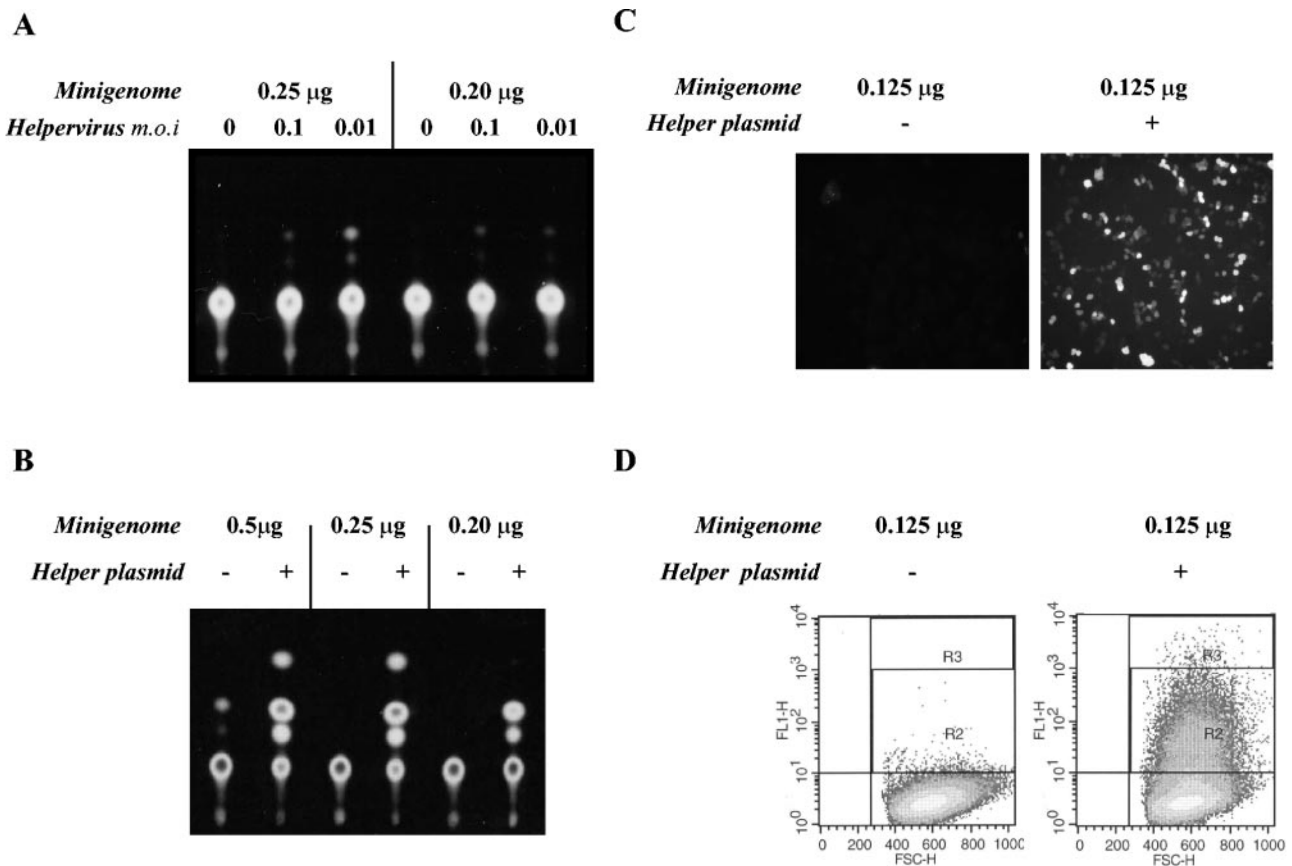

FIG. 2. Optimization of Pol I-driven reporter minigenome systems. (A) Helper virus-driven Pol I-CAT minigenome transcription. Decreasing amounts of the Pol I-driven vRNA-oriented CAT minigenome were transfected into 293T cells and incubated for 24 h at 37°C with 5% CO_2 prior to infection with an MOI of 0, 0.1, or 0.01. Cells were incubated for a further 72 h at 37°C with 5% CO_2 prior to lysis and an assay for CAT activity. CAT activity was determined by incubating 20% of the total cell lysate overnight in the presence of chloramphenicol and acetyl coenzyme A. (B) Helper plasmid-driven Pol I-CAT minigenome transcription. Decreasing amounts of the Pol I-driven vRNA-oriented CAT minigenome and standard amounts of helper plasmid (1.0 μg of NP, 0.5 μg of VP35, 0.3 μg of VP30, and 1.0 μg of L) were transfected into 293T cells and incubated for 48 h at 37°C with 5% CO_2. The cells were then lysed and assayed for CAT activity by incubation of 5% of the total cell lysate for 2 h in the presence of chloramphenicol and acetyl coenzyme A. (C) Helper plasmid-driven Pol I-GFP minigenome transcription, as shown by UV fluorescence. Samples were transfected as described for panel B and were analyzed after 24 h. Data are shown for the transfection of 0.125 μg of minigenome, which yielded optimal results when data were analyzed by UV microscopy. (D) Helper plasmid-driven Pol I-GFP minigenome transcription, as shown by FACS. Samples were prepared as described for panel B. Data are shown for the transfection of 0.125 μg of minigenome, which yielded optimal results when data were analyzed by FACS analysis.

However, this process appears to be much less efficient in the REBOV Pol I-driven system than the recognition of viral promoter elements contained in the noncoding regions by the viral RNP complex proteins, thus allowing a high level of reporter activity in the absence of an appreciable background. As shown previously with minigenome systems for other viruses (13, 22), the plasmid-driven REBOV Pol I minigenome system provided superior reporter expression compared to the helper virus-driven system (Fig. 2A versus B).

We next tried to determine if we could replicate the results of the Pol I-driven CAT minigenome system by using an alternative reporter. Therefore, the same protocol as that used for the helper plasmid-driven CAT minigenome experiment was followed, but with a vRNA-oriented Pol I-driven GFP construct. 293T cells were transfected with various amounts of Pol I-driven GFP minigenome plasmid DNA in the presence or absence of helper plasmids, and GFP expression was monitored by UV microscopy and fluorescence-activated cell sorting (FACS) analysis (Fig. 2C and D). It was determined for this construct that even 125 ng of input minigenome still provided a very high signal intensity in a large proportion of the cell population in the absence of an appreciable background. These data indicate that the results obtained with this system are reproducible when different reporter protein activities are used as an indication of minigenome transcription.

Analysis of Pol I-driven minigenome replication. In the initial experiments, all reporter cassettes were cloned in the vRNA orientation, meaning that the reporter gene was in an antisense orientation on the primary Pol I or T7 transcript. In order to confirm that replication, in addition to the viral transcription shown by the methods described above, was occurring, we cloned the reporter transcription cassette in the cRNA orientation. This construct resulted in a Pol I reporter gene transcript in which the reporter gene was in the sense orien-

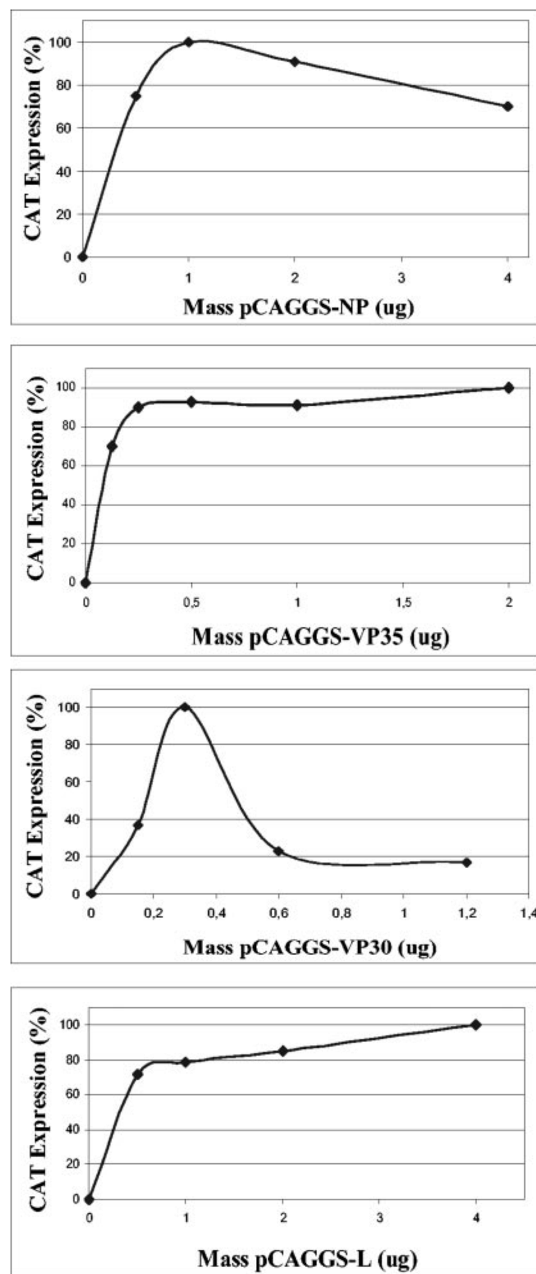

FIG. 3. Optimization of input helper plasmid. Various amounts of helper plasmid were transfected along with 250 ng of Pol I-driven vRNA-oriented CAT minigenome into 293T cells and incubated for 24 h at 37°C with 5% CO_2. The cells were incubated for a further 48 h at 37°C with 5% CO_2 prior to lysis and an assay for CAT activity. CAT activity was determined by incubating 5% of the total cell lysate for 2 h in the presence of chloramphenicol and acetyl coenzyme A.

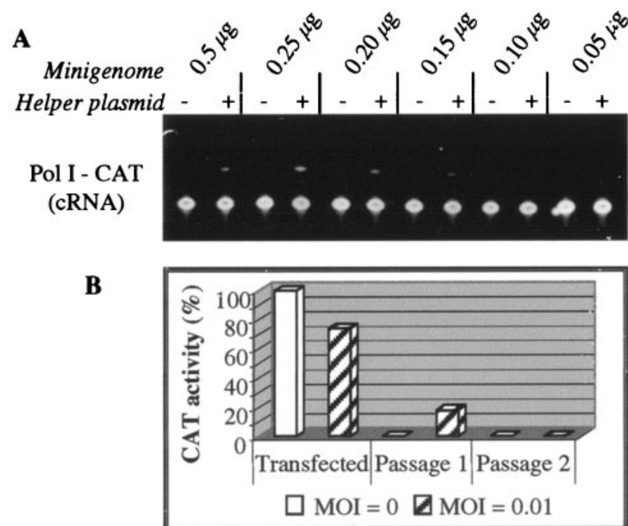

FIG. 4. Demonstration of minigenome replication and packaging. (A) cRNA-oriented Pol I-driven minigenome replication. Various amounts of a Pol I-driven cRNA-oriented CAT minigenome were cotransfected along with standard amounts of each helper plasmid (1.0 μg of NP, 0.5 μg of VP35, 0.3 μg of VP30, and 1.0 μg of L) into 293T cells. The cultures were incubated at 37°C with 5% CO_2 for 48 h prior to harvesting of the cell lysates. For determinations of CAT activity, 5% of the total cell lysate was incubated for 2 h in the presence of chloramphenicol and acetyl coenzyme A. (B) Minigenome packaging into virions. After transfection of 0.25 μg of the vRNA-oriented Pol I-driven CAT minigenome and standard amounts of helper plasmids (1.0 μg of NP, 0.5 μg of VP35, 0.3 μg of VP30, and 1.0 μg of L) into 293T cells, cultures were incubated for 24 h at 37°C with 5% CO_2 and subsequently infected with an MOI of 0.01. Following a further incubation for 72 h at 37°C with 5% CO_2, the cell lysates were harvested and 2 ml of undiluted supernatant was transferred to fresh monolayers. This procedure was repeated twice, with the CAT activities of cell lysates being determined after each passage. For determinations of CAT activity, 5% of the total cell lysate from each passage was incubated for 2 h in the presence of chloramphenicol and acetyl coenzyme A.

tation and flanked by cRNA noncoding regions (Fig. 1). This cRNA-like transcript would have to first be replicated in order to form a vRNA-like molecule and thus be functional for transcription. Thus, the reporter protein activity in this system is dependent on the ability of the minigenome transcript to be replicated (cRNA→vRNA) by the RNP complex proteins. In addition, previous results obtained with the T7-driven ZEBOV infectious clone system showed enhanced virus rescue from a cRNA-oriented genome (27). Therefore, we were interested in determining whether enhanced reporter activity could be obtained by use of a cRNA-oriented minigenome construct. 293T cells were transfected with various amounts of the cRNA-oriented minigenome and with established concentrations of helper plasmids. Surprisingly, the CAT activities measured after 48 h (Fig. 4A) were universally lower than those of the vRNA-oriented constructs (Fig. 2B) when equivalent masses of minigenome and helper plasmids were used for transfections.

Detection of Pol I-driven minigenome packaging. The final element of the successful establishment of a minigenome system is the demonstration of the packaging of minigenome transcripts into virus particles. This can be demonstrated by the ability of reporter activity to be passaged from an initial culture to fresh cell monolayers. 293T cells were initially transfected with both the vRNA-oriented Pol I-driven CAT minigenome and the necessary helper plasmids. This resulted in the efficient transcription of vRNA-like reporter molecules, as indicated by a high CAT activity (Fig. 4B). In the absence of an infecting helper virus, no reporter activity could be passaged from the initial culture to subsequent cell monolayers, despite the presence of higher levels of starting CAT activity in these cultures than in those that were infected (Fig. 4B). However, a REBOV

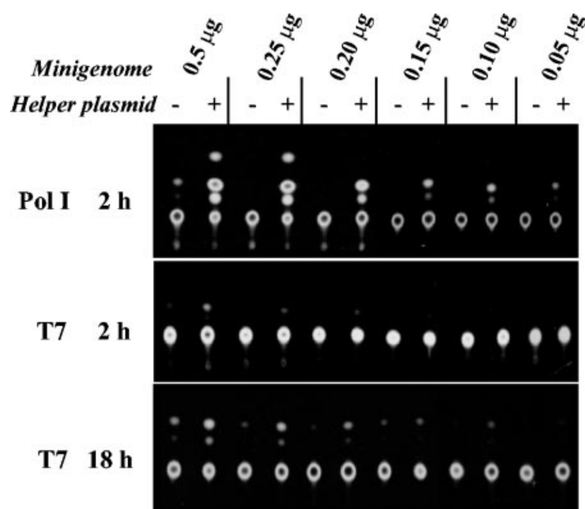

FIG. 5. Comparison of Pol I- and T7-mediated transcription of CAT reporter minigenomes. Various amounts of a T7- or Pol I-driven CAT minigenome were transfected along with standard amounts of helper plasmids (1.0 μg of NP, 0.5 μg of VP35, 0.3 μg of VP30, and 1.0 μg of L). The relative amounts of reporter expression produced by the two systems were compared after the incubation of 5% of the total cell lysate in the presence of chloramphenicol and acetyl coenzyme A for 2 h. In order to obtain more comparable signals, we also incubated the T7-driven minigenome lysates for 18 h.

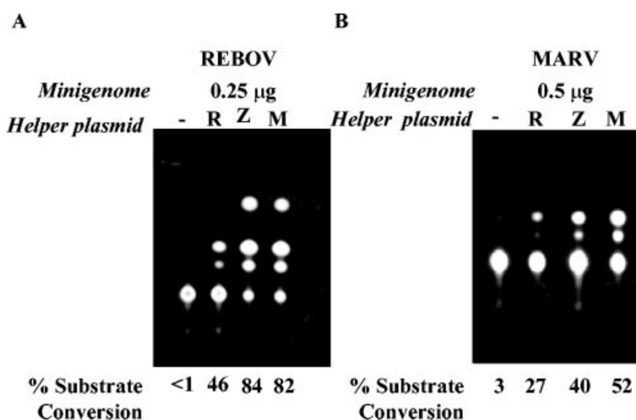

FIG. 6. Comparison of minigenome transcription by RNP complex components from heterologous sources. (A) For the REBOV minigenome system, 0.25 μg of the vRNA-oriented Pol I-driven minigenome was cotransfected along with standard amounts of pCAGGS helper plasmids (1.0 μg of NP, 0.5 μg of VP35, 0.3 μg of VP30, and 1.0 μg of L) derived from either REBOV, ZEBOV, or MARV. Transfected 293T cultures were then incubated for 48 h at 37°C with 5% CO_2 and were assayed for CAT activity by incubation of 5% of the total cell lysate in the presence of chloramphenicol and acetyl coenzyme A for 2 h. (B) For the MARV minigenome system, 0.5 μg of the vRNA-oriented T7-driven minigenome was cotransfected along with standard amounts of helper plasmids derived from REBOV, ZEBOV, or MARV. Transfected 293T cultures were then incubated for 48 h at 37°C with 5% CO_2 and were assayed for CAT activity by incubation of 5% of the total cell lysate in the presence of chloramphenicol and acetyl coenzyme A for 2 h.

infection 24 h after transfection of the minigenome plasmid and the RNP complex protein-encoding plasmids was able to transfer reporter activity to fresh cell cultures. After the first passage, the reporter activity was equal to 16% of the CAT activity observed in the initial culture. By the second passage, the CAT activity had dropped below detectable levels. Thus, the packaging of minigenome vRNA occurred, indicating the presence of the minimal necessary *cis*-acting signals for packaging within the noncoding genome regions flanking the reporter gene. The efficacy of packaging was low but comparable to that in previously published systems (10, 13).

Comparison of transcription in Pol I- and T7-driven systems. Encouraged by the potency of the Pol I-driven minigenome system, we compared Pol I- versus T7-driven REBOV minigenome rescue. In order to assess this, we determined the reporter expression from CAT-containing minigenomes produced according to these two strategies, in the presence or absence of the necessary helper plasmids (Fig. 5). Surprisingly, T7-driven REBOV minigenomes, transfected in an amount which had been found to produce very strong CAT reporter signals (i.e., 250 ng) with the Pol I-driven system after only 2 h of incubation, produced only weak CAT signals (Fig. 5). These signals could be enhanced by incubation for up to 18 h, but even at this time point the levels of reporter activity did not reach those obtained after only 2 h by the Pol I-driven system. In addition, after the prolonged incubation required to obtain strong reporter signals with the T7-driven REBOV minigenome, substantial levels of background became apparent. Background reporter expression was not normally observed with the Pol I-driven minigenome system when <0.5 μg of minigenome was used (Fig. 2B). In addition, the effect of additional G residues was investigated, with no notable effect on reporter activity (data not shown). Thus, in the case of REBOV, the Pol I-driven minigenome system seems to be more potent than the T7-driven system, regardless of the incorporation of upstream G residues.

Pol I-driven minigenome transcription by heterologous helper plasmids. Previous attempts have failed to detect reporter gene expression of MARV and ZEBOV minigenomes with heterologous helper plasmids (26). In order to revisit this issue, we cotransfected 293T cells with optimized concentrations of the Pol I-driven REBOV minigenome plasmid (0.25 μg) and MARV-, ZEBOV-, or REBOV-derived RNP complex protein helper plasmids (NP, 1.0 μg; VP35, 0.5 μg; VP30, 0.3 μg; L, 1.0 μg). The cells were harvested and CAT activity was measured 48 h later (Fig. 6A). Transcription occurred at a similar level with either the MARV or ZEBOV plasmids, and surprisingly, in both cases it was observed that these heterologous RNP complex proteins were able to mediate a higher level of reporter transcription than the REBOV proteins (Fig. 6A). A similar result was also obtained with a T7-driven MARV minigenome system in which MARV helper plasmids produced a slightly higher level of reporter activity than did ZEBOV plasmids (Fig. 6B). However, helper plasmids from both of these viruses produced much higher levels of reporter expression than did those expressing REBOV RNP complex proteins. This clearly indicates the ability of the RNP complex to recognize appropriate signals on heterologous RNA.

DISCUSSION

This study describes the establishment of Pol I- and T7-driven minigenome systems for REBOV. It is the first pub-

lished example demonstrating the successful use of the Pol I transcription strategy for a cytoplasmically replicating nonsegmented negative-strand RNA virus (28).

We have demonstrated that transcription and replication of Pol I-generated REBOV minigenomes by corresponding viral RNP complex proteins can be achieved and that these functions yield the expression of either a CAT (Fig. 2A and B) or a GFP (Fig. 2C and D) reporter from the corresponding vRNA-oriented minigenome construct. In addition, the RNP complex proteins may be delivered either through helper virus infection of minigenome-transfected cells with virus particles (Fig. 2A) or through cotransfection of individual expression plasmids for each of the four proteins, NP, VP35, VP30, and L, required to mediate EBOV transcription and replication (Fig. 2B). In addition to viral protein-mediated transcription, we were also able to confirm that the replication of cRNA-like minigenome transcripts can occur within this system (Fig. 4A). However, the levels of reporter obtained with a cRNA-oriented construct were substantially lower than those achieved by use of a similar minigenome containing the reporter gene in the antisense orientation (Fig. 2B and 4A). This is in contrast to the results obtained during the development of the ZEBOV infectious clone system (27), but note that the efficiency of virus rescue within this system reflects a much more complex process.

Finally, we have confirmed that the packaging of REBOV minigenomes into progeny virions is possible within the minigenome rescue system, based on the ability of supernatants from REBOV-infected cells that were previously transfected with minigenome and helper plasmids to transfer reporter activity to fresh cell cultures (Fig. 4B). In this system, the transfer of reporter activity occurred at a low level, and thus, transfer was only observed for a single passage. This likely reflects both the small number of REBOV progeny that would be expected during the time frame of these experiments and the improbability of both a wild-type REBOV and a reporter minigenome-containing virus infecting a single cell, as would be required for transfer through a second passage, since we did not reintroduce virion protein components into these cell cultures. It is important that the transfer of reporter protein activity did not occur in the absence of virus infection since this step is required in order to supply the remaining structural proteins that are necessary for virion assembly. This was despite the high initial levels of reporter activity in these samples, which exceeded those produced by the infected cells (Fig. 4B). This clearly demonstrates the absence of passive transfer of either transfected plasmid DNA or minigenome RNA in transfected cells.

In order to evaluate the potential of this newly developed Pol I-driven minigenome system as an alternative to the more common T7-driven system, we compared the REBOV Pol I-driven minigenome to one developed based on the T7-driven systems previously established for ZEBOV and MARV (3, 25, 26) (Fig. 5). When equal concentrations of minigenome plasmids were transfected along with the required helper plasmids, the Pol I-driven construct produced much stronger reporter gene expression levels, while in the absence of helper plasmids, the level of CAT expression observed with the Pol I-driven system was less than that for an equivalent mass of T7-driven minigenome. Based on these data, Pol I appears to be an attractive alternative to T7 for the development of minigenome systems, as this system not only eliminates the need for two inefficient steps associated with the T7 system, ribozyme cleavage and exogenous polymerase introduction, but for REBOV, also provides much stronger positive reporter signals and lower levels of background reporter expression.

Existing minigenome rescue and infectious clone systems for members of the family *Filoviridae* have all been based on initial transcription by the bacteriophage T7 polymerase (3, 25–27, 36). However, this system requires the introduction of the exogenous T7 polymerase, and while this can be achieved in several ways, including recombinant vaccinia virus infection (14) and transient or stable expression from plasmid DNA (27, 35, 36), this step has the potential to limit the system's efficiency due to technical difficulties with achieving the expression of T7 in all cells within the culture. In contrast, Pol I is a eukaryotic host cell polymerase which is normally localized to the nucleoli, where it is responsible for the transcription of the 5.8S, 28S, and 18S rRNA transcripts (synthesized as a 45S pre-rRNA precursor molecule). This provides a substantial advantage in terms of the development of minigenome systems, since it alleviates the need to supply the polymerase in *trans*. While the localization of Pol I is one potential limitation of this system for its application to viruses which replicate exclusively in the cytoplasm, such as filoviruses, it was previously employed very successfully for the development of minigenomes for several members of the family *Bunyaviridae* (9, 11, 13) as well as for the *Arenaviridae* (22). Together with our data, this indicates that Pol I-driven transcription may be a broadly applicable system for minigenome rescue with cytoplasmically replicating viruses. However, the ability of Pol I to mediate the synthesis of transcripts exceeding 3.4 kb in length has not yet been demonstrated (31) and may represent a potential limitation of the utility of this system for the development of infectious clone systems for many viruses.

Another substantial limitation of the traditional T7-driven system involves the addition of extra nucleotides to the 5′ and 3′ ends of T7 transcripts as a result of its complex transcription initiation and termination (19, 20). These additional nucleotides, because of their potential to interfere with secondary structure formation within the noncoding regions and the binding of the viral RNA polymerase, require the incorporation of an HDV ribozyme sequence at the 3′ end of the transcription cassette (30). While cleavage by the HDV ribozyme produces an authentic 3′ terminus, the efficiency with which ribozyme cleavage of the transcript takes place is very low and therefore has further potential to limit the production of functional minigenome transcripts within this system (22). These issues are addressed in the Pol I-driven minigenome system, which exhibits very clearly defined initiation and termination sites (12, 38). As a result, additional nucleotides are not added to either end of the transcript, thus precluding the need for ribozyme cleavage. Based on these properties, we believe that Pol I presents an attractive alternative to T7 for future minigenome system development for filoviruses as well as for other cytoplasmically replicating members of *Mononegavirales*.

Using previously developed T7-based minigenome systems for ZEBOV and MARV, others observed no reporter activity when RNP complex proteins were supplied by the infection of minigenome-transfected cell cultures with other filovirus fam-

ily members (26). This was despite strong positive signals obtained with infection by the same virus from which the minigenome was derived. In contrast, our recent data indicate that with the infectious clone system for ZEBOV, virus rescue can be obtained with some combinations of heterologous helper plasmids (35) supplied from REBOV or MARV. Similarly, the data presented here clearly indicate that both ZEBOV- and MARV-derived helper plasmids are capable of supporting transcription and replication in a REBOV minigenome system (Fig. 6). In addition, we observed that either MARV or ZEBOV is capable of supporting reporter gene transcription at a consistently higher level than the REBOV helper plasmids themselves, despite being expressed under the control of the same promoter. A very similar trend was also demonstrated with a T7-driven MARV minigenome system, suggesting that pathogenic members of the filovirus family may be more transcriptionally active than their apathogenic counterparts. Such a difference may ultimately contribute to the differing degrees of pathogenicity observed among these family members.

These data emphasize the importance of studying apathogenic viruses, not only as model systems, but also for the insight that they can provide into aspects of pathogenesis. In particular, the development of filovirus reverse genetics for REBOV would offer us the opportunity to compare this species to other more virulent ones, particularly ZEBOV, for which mortality rates can approach 90% (5, 33) and for which an infectious clone system has already been developed (27, 36). Such experiments would have the potential to provide insights into the identification of factors that contribute to pathogenic differences by comparing complementary mutations in these two systems. It is also possible that the use of an apathogenic REBOV infectious clone as the background into which mutations will be introduced may improve our ability to detect the influence of individual genes or sequences involved in pathogenesis beyond what is possible by use of the pathogenic ZEBOV system (16, 17, 34). While information obtained during the development of our minigenome system for REBOV provides us with valuable data to assist with future attempts to establish such an infectious clone system, minigenome systems also represent powerful tools in their own right, allowing us to investigate factors affecting virus encapsidation, transcription, replication, and packaging while eliminating biosafety constraints required for work with infectious systems.

ACKNOWLEDGMENTS

We thank Ute Ströher for providing the *Reston ebolavirus* RNA, Thomas G. Ksiazek and Pierre E. Rollin for providing us with *Ebolavirus* strains, John Rose for providing the VSV genome-containing plasmid, and Yoshihiro Kawaoka for providing the pCAGGS-T7 expression vector. We are also grateful to Hideki Ebihara and Thomas Hoenen for critical readings of the manuscript and for their valuable insights and to Markus Czub and Steven Jones for training on the phosphorimager and FACS analyzer, respectively.

This work was supported by a grant from the Canadian Institutes of Health Research (MOP 43921) awarded to H.F. and by a scholarship of the Natural Sciences and Engineering Research Council of Canada (CGSD2-302937-2004) awarded to A.G. These studies were performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy for A.G.

REFERENCES

1. **Centers for Disease Control.** 1990. Filovirus infection in animal handlers. Morb. Mortal. Wkly. Rep. **39:**221.
2. **Centers for Disease Control.** 1990. Evidence of filovirus infection in an animal caretaker in a research/service facility. Morb. Mortal. Wkly. Rep. **39:**296–297.
3. **Crary, S., J. Towner, J. Honig, T. Shoemaker, and S. Nichol.** 2003. Analysis of the role of predicted RNA secondary structures in Ebola virus replication. Virology **306:**210–218.
4. **Feldmann, H., T. W. Geisbert, P. B. Jahrling, H.-D. Klenk, S. V. Netesov, C. J. Peters, A. Sanchez, R. Swanepoel, and V. E. Volchkov.** 2004. *Filoviridae. In* C. M.Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), Virus taxonomy: eighth report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, Calif.
5. **Feldmann, H., S. Jones, H.-D. Klenk, and H. Schnittler.** 2003. Ebola virus: from discovery to vaccine. Nat. Rev. Immunol. **3:**677–685.
6. **Feldmann, H., and M. Kiley.** 1999. Classification, structure, and replication of filoviruses. Curr. Top. Microbiol. Immunol. **235:**1–21.
7. **Feldmann, H., V. Volchkov, U. Ströher, V. Volchkova, and H.-D. Klenk.** 2001. Biosynthesis and role of filovirus glycoprotein. J. Gen. Virol. **82:**2839–2848.
8. **Fisher-Hoch, S., and L. Brammer.** 1992. Pathogenic potential of filoviruses: role of geographic origin of primate host and virus strain. J. Infect. Dis. **166:**753–763.
9. **Flick, K., J. Hooper, C. Schmaljohn, R. Pettersson, H. Feldmann, and R. Flick.** 2003. Rescue of Hantaan virus minigenomes. Virology **306:**219–224.
10. **Flick, K., A. Katz, A. Överby, H. Feldmann, R. Pettersson, and R. Flick.** 2004. Functional analysis of the non-coding regions of the Uukuniemi virus (*Bunyaviridae*) RNA segments. J. Virol. **78:**11726–11738.
11. **Flick, R., K. Flick, H. Feldmann, and F. Elgh.** 2003. Reverse genetics for Crimean-Congo hemorrhagic fever virus. J. Virol. **77:**5997–6006.
12. **Flick, R., and G. Hobom.** 1999. Transient bicistronic vRNA segments for indirect selection of recombinant influenza viruses. Virology **262:**93–103.
13. **Flick, R., and R. Pettersson.** 2001. Reverse genetics system for Uukuniemi virus (*Bunyaviridae*): RNA polymerase I-catalyzed expression of chimeric viral RNAs. J. Virol. **75:**1643–1655.
14. **Fuerst, T., E. Niles, F. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
15. **Groseth, A., U. Stroher, S. Theriault, and H. Feldmann.** 2002. Molecular characterization of an isolate from the 1989/90 epizootic of Ebola virus Reston among macaques imported into the United States. Virus Res. **87:** 155–163.
16. **Hatta, M., P. Halfmann, K. Wells, and Y. Kawaoka.** 2002. Human influenza A viral genes responsible for the restriction of its replication in duck intestine. Virology **295:**250–255.
17. **Ito, T., H. Goto, E. Yamamoto, H. Tanaka, M. Takeuchi, M. Kuwayama, Y. Kawaoka, and K. Otsuki.** 2001. Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J. Virol. **75:**4439–4443.
18. **Jahrling, P., T. Geisbert, D. Dalgard, E. Johnson, T. Ksiazek, W. Hall, and C. Peters.** 1990. Preliminary sequence report: isolation of Ebola virus from monkeys imported to USA. Lancet **335:**502–505.
19. **Kato, A., Y. Sakai, T. Shioda, T. Kondo, M. Nakanishi, and Y. Nagai.** 1996. Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. Genes Cells **1:**569–579.
20. **Krupp, G.** 1988. RNA synthesis: strategies for the use of bacteriophage RNA polymerases. Gene **72:**75–89.
21. **Laboratory Centre for Disease Control.** 1996. Laboratory biosafety guidelines, 2nd ed. Health Canada, Ottawa, Ontario, Canada.
22. **Lee, K., I. Novella, M. Teng, M. Oldstone, and J. de La Torre.** 2000. NP and L proteins of lymphocytic choriomeningitis virus (LCMV) are sufficient for efficient transcription and replication of LCMV genomic RNA analogs. J. Virol. **74:**3470–3477.
23. **Miranda, M., T. Ksiazek, T. Retuya, A. Khan, A. Sanchez, C. Fulhorst, P. Rollin, A. Calaor, D. Manalo, M. Roces, M. Dayrit, and C. Peters.** 1999. Epidemiology of Ebola (subtype Reston) virus in the Philippines, 1996. J. Infect. Dis. **179:**S115–S119.
24. **Miranda, M., M. White, M. Dayrit, C. Hayes, T. Ksiazek, and J. Burans.** 1991. Seroepidemiological study of filovirus related to Ebola in the Philippines. Lancet **337:**425–426.
25. **Mühlberger, E., B. Lotfering, H.-D. Klenk, and S. Becker.** 1998. Three of the four nucleocapsid proteins of Marburg virus, NP, VP35, and L, are sufficient to mediate replication and transcription of Marburg virus-specific monocistronic minigenomes. J. Virol. **72:**8756–8764.
26. **Mühlberger, E., M. Weik, V. Volchkov, H.-D. Klenk, and S. Becker.** 1999. Comparison of the transcription and replication strategies of Marburg virus and Ebola virus by using artificial replication systems. J. Virol. **73:**2333–2342.
27. **Neumann, G., H. Feldmann, S. Watanabe, I. Lukashevich, and Y. Kawaoka.** 2002. Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. J. Virol. **76:**406–410.
28. **Neumann, G., M. Whitt, and Y. Kawaoka.** 2002. A decade after the generation of a negative-sense RNA virus from cloned cDNA—what have we learned? J. Gen. Virol. **83:**2635–2662.

29. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193–199.
30. **Perrotta, A., and M. Been.** 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature **350:**434–436.
31. **Pinschewer, D., M. Perez, A. Sanchez, and J. de la Torre.** 2003. Recombinant lymphocytic choriomeningitis virus expressing vesicular stomatitis virus glycoprotein. Proc. Natl. Acad. Sci. USA **100:**7895–7900.
32. **Rollin, P., R. Williams, D. Bressler, S. Pearsom, M. Cottingham, G. Pucak, A. Sanchez, S. Trappier, R. Peters, P. Geer, S. Zaki, T. Demarcus, K. Hendricks, M. Kelley, D. Simpson, T. Geisbert, P. Jahrling, C. Peters, and T. Ksizek.** 1999. Ebola (subtype Reston) virus among quarantined nonhuman primates recently imported from the Philippines to the United States. J. Infect. Dis. **179:**S108–S114.
33. **Sanchez, A., A. Khan, S. Zaki, G. Nabel, T. Ksiazek, and C. Peters.** 2001. Filoviridae: Marburg and Ebola viruses, p. 1279–1304. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams and Wilkins, Philadelphia, Pa.
34. **Subbarao, K., W. London, and B. Murphy.** 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J. Virol. **67:**1761–1764.
35. **Theriault, S., A. Groseth, G. Neumann, and H. Feldmann.** 2004. Rescue of Ebola virus from cDNA using heterologous support proteins. Virus Res. **106:**43–50.
36. **Volchkov, V., V. Volchkova, E. Mühlberger, L. Kolesnikova, M. Weik, O. Dolnik, and H.-D. Klenk.** 2001. Recovery of infectious Ebola virus from cDNA: transcriptional RNA editing of the GP gene controls viral cytotoxicity. Science **291:**1965–1969.
37. **World Health Organization.** 1992. Viral haemorrhagic fever in imported monkeys. Wkly. Epidemiol. Rec. **24:**183.
38. **Zobel, A., G. Neumann, and G. Hobom.** 1993. RNA polymerase I catalyzed transcription of insert viral cDNA. Nucleic Acids Res. **21:**3607–3614.