Ebola Virus Defective Interfering Particles and Persistent Infection

Philippe Calain, Martha C. Monroe, and Stuart T. Nichol

Special Pathogens Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Mailstop G14, 1600 Clifton Road, N.E., Atlanta, Georgia 30329-4018

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Ebola virus (Zaire subtype) is associated with high mortality disease outbreaks that commonly involve human to human transmission. Surviving patients can show evidence of prolonged virus persistence. The potential for Ebola virus to generate defective interfering (DI) particles and establish persistent infections in tissue culture was investigated. It was found that serial undiluted virus passages quickly resulted in production of an evolving population of virus minireplicons possessing both deletion and copyback type DI genome rearrangements. The tenth undiluted virus passage resulted in the establishment of virus persistently infected cell lines. Following one or two crises, these cells were stably maintained for several months with continuous shedding of infectious virus. An analysis of the estimated genome lengths of a selected set of the Ebola virus minireplicons and standard filoviruses revealed no obvious genome length rule, such as "the rule of six" found for the phylogenetically related Paramyxovirinae subfamily viruses. Minimal promoters for Ebola virus replication were found to be contained within 156 and 177 nucleotide regions of the genomic and antigenomic RNA 3′ termini, respectively, based on the length of authentic termini retained in the naturally occurring minireplicons analyzed. In addition, using UV-irradiated preparations of virus released from persistently infected cells, it was demonstrated that Ebola virus DI particles could potentially be used as natural minireplicons to assay standard virus support functions.

INTRODUCTION

Viruses of the Filoviridae family include some of the most virulent infectious agents of human and nonhuman primates. They induce acute, rapidly progressing, frequently fatal diseases typically characterized by hemorrhages, shock, and multiorgan involvement (Bwaka et al., 1999). Primary targets appear to be endothelial and macrophage cells, infection of which likely plays an important role in disease development (Feldmann et al., 1996). The filoviruses are enveloped, nonsegmented, negative-stranded RNA viruses with a characteristic filamentous, pleomorphic shape when observed by electron microscopy. Two serologically (Feldmann et al., 1994) and genetically (Sanchez et al., 1993, 1996) distinct filovirus groups, designated Marburg and Ebola, exist in unidentified primary reservoirs probably located in forest areas of tropical Africa (Sanchez et al., 1996; Swanepoel et al., 1996). Phylogenetic analysis of all the currently characterized Ebola viruses, including the most recent isolates from Gabon (Georges-Courbot et al., 1997; Volchkov et al., 1997), reveals four distinct Ebola virus species: Zaire (including Gabon strains), Sudan, Reston, and Côte D'Ivoire.

Defective interfering (DI) particles have been observed in viruses representing nearly all RNA virus families (Perrault, 1981; Holland et al., 1982). They are infectious units containing truncated genomes and therefore require the presence of a full-length nondefective helper virus (called "standard" virus) for their propagation. Conditions of high multiplicity of infection (m.o.i.) favor the amplification of DI particles. Two basic patterns of truncation result in replicons constituting infectious DI particles (Perrault, 1981) (Fig. 1), although more complex structures have been observed (Nichol et al., 1984). In the first pattern, called "deletion defective replicons," the standard genomic 3′ and 5′ termini are retained. Like their standard homologs, deletion defective replicons thus contain two different terminal cis sequences for promoting the synthesis of either antigenomes or genomes. In the pattern called "copyback defective replicons," the molecule is derived exclusively from the genomic 5′-end and both ends are self-complementary. Termini of copy-back replicons therefore only contain one type of replication promoter, the one originally present on standard antigenomes.

Defective-interfering particles are associated with two important phenomena: attenuation and persistence. For decades, it has been known that serial undiluted virus passages can result in virus attenuation in animals, embryonated eggs, or tissue culture cells (Holland et al., 1982; Roux et al., 1991). As mentioned above, these conditions favor the amplification of DI particles. A more obvious link between attenuation and DI particles in tissue culture can be shown if DI particles are directly mixed with homologous standard virus. In a typical in-
terference assay, the presence of DI particles results in attenuation, delay, or absence of the cytopathic effect (CPE) normally associated with the standard virus, and in a decrease in the yield of progeny virus. In some cases, infected cells survive and become long-term persistently infected cell lines. Although it is clear that DI particles...
can play an important role in the initiation and maintenance of persistent infections per se, other factors (ts mutants, interferon, cell membrane alterations, or other cell mutations) can also be involved (Ahmed et al., 1981; Moscona et al., 1993; Segev et al., 1995; Stanners et al., 1977).

Research strategies based on reverse genetics have recently been developed for several nonsegmented negative-stranded RNA viruses. Full-length infectious clones are now available for rabies virus (Schnell et al., 1994), vesicular stomatitis virus (Lawson et al., 1995; Whelan et al., 1995), measles virus (Radecke et al., 1995), Sendai virus (Garcin et al., 1995), respiratory syncytial virus (Collins et al., 1995), and human parainfluenza virus type 3 virus (Hoffman and Banerjee, 1997). Such techniques are obviously important for gaining an understanding of virus/host interactions in cell culture and ultimately in the infected host. As a preliminary approach to reverse genetics of filoviruses, we have carried out a series of experiments that have generated an evolving population of Ebola virus DI particles and a monkey cell line persistently infected with Ebola virus. Detailed analysis of these systems also provides insight into Ebola virus replication and persistence in natural infections.

RESULTS

Ebola virus high multiplicity passage leads to production of virus DI particles and persistently infected cells

An Ebola virus undilute passage series was initiated by infection of cells at an m.o.i. greater than one of a virus stock (designated PO) which had been prepared previously by low m.o.i. passage (see Materials and Methods). A series of 10 undiluted Ebola virus passages (designated P1 to P10) were successfully carried out. From passage 7 until passage 10, the yield of infectious virus particles decreased such that it was no longer possible to ensure an m.o.i. above 1 with the successive inocula. In parallel to this drop in infectious virus yields, the time of appearance of the virus induced CPE also became much delayed. By virus passage P10, the infected cell monolayer remained intact, and on passaging of the cells, no virus-induced CPE was seen up to 32 days postinfection (p.i.). Standard virus particles capable of causing CPE were still present in these P10-infected cells as the supernatant removed from these cells 3 days p.i. yielded 2.6 \times 10^{10} infectious virus particles capable of causing cell death as indicated by their ability to form plaques in a traditional virus plaque assay (Table 1). These data strongly suggest that the lack of CPE observed in cells generating virus P10 was due to generation of virus interfering particles, and not to selection of noncytopathic standard virus. To examine this further, passage 10 virus was assayed for the ability to interfere with standard virus replication as described under Materials and Methods. Cells challenged with standard working stock virus were almost totally protected from undergoing CPE when P10 virus was added to the initial inoculum (Table 1). By comparison, control cells infected with working stock virus alone underwent strong CPE, with most cells being destroyed by 3 days p.i. In addition to protection from CPE, working stock virus yields were reduced by greater than 90% in the cultures infected in the presence of P10 virus (Table 1). The development of virus interference capability correlated with the appearance of virus-defective RNAs detectable by Northern blot analysis (see below). Taken together, these data indicate that DI particles had been generated during the high multiplicity passage series.

Generation of stable Ebola virus persistently infected cell lines

At day 7 p.i., the VeroE6 cells infected with P9 virus (i.e., those yielding P10 virus) were passaged by splitting the cells following trypsinization of the monolayer. Subsequent passaging of the cells continued at approximately 2-week intervals. After four passages, the cell monolayers underwent crisis and exhibited extensive CPE with lysis of approximately 90% of the cells. By convention, cells were designated according to their passage history: “Pix.Cy” where x is the number of cell passages after the P10 infection and y is the number of spontaneous crisis events. Cells remaining after the first crisis recovered and could be stably propagated for four to six additional passages, at which times cell stocks (Pi8.C1 and Pi10.C1) were frozen. From thawed aliquots of the frozen cells, two lineages could be recovered and stably maintained for at least 30 passages. One lineage (Pi9.C2 derived from Pi8.C1) underwent an additional crisis early after resumption of the culture but remained stable thereafter. The other lineage was derived from a sample of Pi10.C1 cells and remained stable upon propagation.

Almost all cells in both cell lineages appeared to be

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Virus yield^a</th>
<th>Percentage yield^b</th>
<th>CPE^c</th>
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<tr>
<td>Standard virus</td>
<td>2.0 \times 10^8</td>
<td>100.0</td>
<td>++ + + +</td>
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<tr>
<td>Standard virus + P10</td>
<td>1.3 \times 10^8</td>
<td>6.5</td>
<td>+/-</td>
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<tr>
<td>P10 stock alone</td>
<td>2.6 \times 10^8</td>
<td>1.3</td>
<td>-</td>
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<td>Mock infected</td>
<td>0.0</td>
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Note. The working stock was used as a source of standard virus (see Materials and Methods). Tiers of the inocula were 1.2 \times 10^5 and 3 \times 10^5 PFU/ml for working stock and P10 virus, respectively.

^a PFU/ml at 3 d.p.i.

^b Percentage yield relative to standard working stock virus.

^c CPE assayed at 3 d.p.i.
persistently infected and expressed viral antigens as determined by immunofluorescence assay using an anti-Ebola polyclonal antibody (Fig. 2A). In addition, virus persistence could also be demonstrated by immunohistochemistry and electron microscopy (data not shown). Persistent virus shedding was demonstrated by titration of virus in the cell supernatants. Typical virus yields early after cell monolayer confluence were between $5 \times 10^4$
and $5 \times 10^5$ PFU/ml, with no obvious difference between the two cell lines. While the viruses released from Pi cells tended to have a small plaque phenotype relative to the starting P0 virus stock, cell infections initiated with picked plaques of these Pi viruses were found to display as extensive CPE as those initiated with P0 virus stocks. This observation would be consistent with the lack of CPE in the persistently infected cells being related to the presence of virus DI particles rather than selection of noncytopathic standard viruses. As expected, these persistently infected cell lines were resistant to homologous virus challenge. For instance, Ebola P0 virus caused extensive CPE in Vero E6 cells, but no CPE in Pi5.C1 cells 5 days postchallenge (data not shown). Extensive CPE was seen in both Vero E6 and Pi5.C1 cells 24 h postinfection with vesicular stomatitis virus (VSV), demonstrating that we had not merely selected Pi cell lines resistant to virus CPE in general. Further, the resistance to superinfection was shown to be Ebola virus specific as Pi24.C1 cells were still resistant to Ebola P0 virus CPE, but were completely susceptible to Marburg virus infection, showing extensive CPE by 8 days post-Marburg virus infection (data not shown).

Detection of Ebola virus defective RNAs in undilute passaged virus and persistently infected cells

The data presented above strongly suggested DI particles had been generated during undilute Ebola virus passage and had allowed initiation and maintenance of Ebola virus persistently infected cells. Two main classes of DI particles have been seen in other nonsegmented negative strand viruses, namely, deletion and copyback DI (see Introduction and Fig. 1 (Perrault, 1981)). In Northern blots, deletion DI RNAs would be expected to hybridize to riboprobes for the standard genome 3' or 5' terminal sequences, but not to probes for central regions of the genome such as the VP40 gene. Copyback DI RNAs would be expected to hybridize to riboprobes for standard genome 5' terminal sequences but not 3' terminal or central genome regions such as the VP40 gene.

To determine whether such deletion or copyback RNA molecules were present in undilute virus passage material or persistently infected cells, total cell RNA from each virus passage and selected persistently infected cell passages was analyzed by Northern blot using plus sense riboprobes to detect standard genome 3' and 5' termini (rNP and rL, respectively, Fig. 3). Prominent subgenomic RNA species were detectable in both the undiluted passage and persistent infection RNA samples, using the 3' and 5' probes. It is unlikely that these subgenomic RNAs represent breakdown products of virus standard genome RNAs as no such subgenomic RNA bands were detected with a VP40 riboprobe, even after considerably longer exposure of the northern blot (data not shown). Subgenomic RNAs were quite prominent by the third undilute (P3) virus passage (Fig. 3), and could actually be detected as early as the P2 passage (data not shown). During the undilute virus passage and

FIG. 3. Northern blot analysis of total RNA from cells infected with Ebola virus passages P0, P3, and P10 and from persistently infected cells after their first (Pi4.C1, Pi25.C1) or second (Pi28.C2) crisis. The left and right panels show total cell probed with rNP and rL probes (see Materials and Methods), respectively. RNA from mock-infected cells are indicated with an m. Straight line indicates origin of migration, and arrow indicates position of full-length nondefective RNA genomes. Size and position of cold transcripts used as molecular weight markers are indicated on the left. Exposure times of the two membranes were chosen so the intensity of nondefective genomes would be approximately comparable between the two panels.

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persistently infected cell passage series, the amount of standard virus RNA detectable cycled up and down, and the population of subgenomic RNA molecules appeared to progressively evolve toward lower molecular weight species. Early passages (from Pi1 to Pi3) of the persistently infected cells (data not shown) displayed the same subgenomic RNA pattern as seen in the undilute virus passage 10 material (lane P10, Fig. 3). The differences observed in the pattern of subgenomic RNA species detected using probes rL and rNP suggested that both deletion (reacting with both rL and rNP) and copy-back (reacting only with rL) variants were present in the RNA population. Immediately following the first cell crisis (Pi4.C1), all defective RNA species appeared to be replaced by RNAs of approximately 1 kb in size, detected by both probes rL and rNP (see lane Pi4.C1, Fig. 3). As described below, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that this RNA population is actually heterogeneous, comprising at least two distinct species of both virus deletion and copy-back defective RNA species.

Northern blot analysis of RNA from the later passages of Pi cells (lanes Pi25.C1 and Pi28.C2 in probe rL panel, Fig. 3) showed differences in the migration pattern of subgenomic RNA molecules, compared to Pi4.C1 samples. However, the most dramatic change is the disappearance of species reacting with the rNP probe in lineage PiC1, and the emergence of new prominent species reacting exclusively with probe rL in both lineages. This suggested that copy-back defective genomes progressively outcompeted deletion defective RNA species during this persistently infected cell passage series.

Analysis of Ebola virus defective RNA species by RT-PCR

A series of RT-PCR assays were designed for the detection of Ebola virus deletion (DEL) and copyback (CB) defective RNAs (illustrated in Fig. 1). The RT primer targeted the + sense (antigenomic) copy of the defective RNAs and primed the cDNA synthesis. Then a pair of PCR primers were used to amplify the cDNA region which contained the DEL or CB genome rearrangement. The strategy of detecting molecules of antigenomic (as opposed to genomic) polarity was chosen in order to prime and initiate reverse transcription outside of any region of intramolecular terminal complementarity (Calain et al., 1992). Three types of CB reactions (CBa, CBb, and CBc) were designed in order to increase the chances of detecting CB molecules generated by different virus polymerase premature termination and reinitiation events (Fig. 1).

RT-PCR analysis of total RNA preparations from selected virus and persistently infected cell passages was carried out using the DEL, CBa, CBb, or CBc primer sets (Fig. 4). RNA from an unrelated preparation of Ebola virions (Stock 807346) was also included in the analysis. Distinct PCR products could be detected exclusively from the samples that had been shown to contain defective RNA molecules by Northern-blot analysis, and also
from the 807346 virion RNA preparation (Fig. 4). No amplification products were generated by equivalent RNA amounts from cells infected by P0 (acute infection) virus. All the reactions, except CBc yielded PCR products. For RNA from infected cells, all detectable PCR products were less than 2.5 kb in size.

The most prominent bands generated by reactions DEL, CBa, and CBb were eluted from the gel and sequenced either directly or following TA cloning. Seven distinct sequences could be unambiguously determined. Assuming classical structures, the full-length pattern of the corresponding defective molecules was extrapolated from the position of the DI-like genome rearrangement (Fig. 5). Of the seven characterized virus defective RNA molecules, two had deletion-type genomes and five had copy-back-type genomes. All were derived from persistently infected Pi6.C1 cells, except 15.3, which is a copy-back molecule amplified from virion RNA 807346.

In order to rule out that these molecules merely represented PCR artifacts, i.e., recombinant molecules generated by reverse transcriptase or Taq polymerase jumping events, the actual presence of the recombination sites in the RNA template populations was confirmed in four selected cases (del2.5, del3.3, cb6.2, and cb10) using new sets of specific primers. Basically, the PCR primers 2 and 3 shown in Fig. 1, were substituted with new primers designed to span the virus RNA template recombination points, such that the site of the RNA template breakpoint will be included within the primer, i.e., to detect both antigenomic and genomic defective RNAs. As expected, specific PCR products were generated from Pi6.C1 cell RNA and were absent from mock or acutely infected cells (Fig. 6). Both antigenomic and genomic sense defective RNAs could be detected. PCR products representing both sides of the RNA recombination points had the expected sizes, confirming that species del2.5 and del3.3 were classical deletion defective RNA genomes. For copy-back species, only antigenomic copies could be confirmed despite trying a variety of RT primer annealing conditions. This is likely due to the difficulty in RT priming within the

FIG. 5. Deduced structure of seven Ebola virus DI particles. The structures of two deletion types and five copyback types of Ebola virus DI are shown in the antigenomic (predominantly plus sense) form. The antigenome (plus sense) RNA regions are shown in white and the genome (minus sense) RNA is shown in black. The white box indicates the 3′ promoter present on standard virus plus sense. The black triangle and box indicate the 5′ ends of the standard virus plus and minus sense RNAs, respectively, which represent the complement of the promoters (see Fig. 1). Nucleotide positions of DI breakpoints are numbered relative to the standard virus genome 3′ terminus and are indicated below each type of molecule. The hatched region represents the copied area of the nascent RNA, which is capable of forming complementary panhandle structures with the other terminus of the molecule.
panhandle structure when trying to amplify cDNA copies off the genome sense RNA templates.

**Ebola virus defective particles can be passaged and used as a source of natural minireplicons**

While the data presented in the previous sections would strongly suggest that the defective RNAs characterized must be released from each infection and passaged to the next, all of the analysis was carried out using infected cell RNA. So to directly demonstrate passage of these infectious defective virus particles, virions were purified from the supernatant of Pi14.C1 and Pi25.C2 cells and inoculated onto fresh VeroE6 cells (see Materials and Methods). These cells were then probed for the presence of DI species del2.5, del3.3, and cb6.2 using the RT-PCR systems developed for the precise detection of antigenomic copies of these molecules. The specific PCR products were not detectable at 1 h p.i. (ruling out any signal from potential carry over of any noninfectious free virus nucleocapsid or unencapsidated virus RNA), but was clearly detected 24 h p.i., demonstrating that a new infectious cycle of defective particles had been initiated with the virions purified from the Pi cell supernatants (data not shown). In addition, *de novo* generation of del2.5, del3.3, and cb6.2 defective replicons was not observed with cells infected with an inoculum of P0 standard virions. Therefore, it was concluded that the two lineages of Pi cells propagated and shed at least three of the defective particles represented in Fig. 5.

One of the initial goals of this study was to see if it was possible to generate Ebola virus DI particles which could be used as natural minireplicons to assay standard virus support functions. Such molecules would represent a useful tool during the development of a reverse genetics system for Ebola virus. Despite several attempts, we were unable to produce Ebola virus DI particles free of standard virus particles using various gradient ultracentrifugation methods. However, by exploiting the large genome size difference between standard (19 kb) and DI virus particles, we were able to remove standard virus activity from DI virus-enriched supernatants by UV irradiation. The UV dose was empirically determined to inactivate most of the endogenous standard virus, while leaving intact the much smaller defective RNA molecules previously characterized. The experiment consisted of

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**FIG. 6.** Specific PCR detection of Ebola DI species del2.5, del3.3, cb6.2, and cb10. Analysis of the PCR products generated using primers specific for the detection of the + or – sense forms of del2.5, del3.3, cb6.2, and cb10 DI RNAs. MW represents molecular weight markers composed of ΦX174 DNA cleaved with *Hae*III. The sizes of the specific products expected for classical DI structures are indicated under the lanes where they appear. RNA samples analyzed were extracted from mock-infected cells (m), from cells infected with Ebola virus P0 or with virus stock 807346, and from persistently infected cells Pi6.C1.
using a preparation of ancestral (P0) standard virus to support replication of DI particles present in UV-irradiated virions purified from persistently infected cells (Pi25.C2) (Fig. 7). Replication of copyback 6.2 DI RNAs could be detected 3 days p.i. using a RNase protection assay (Fig. 7A), and after 1 day with a specific RT-PCR (not shown). Replication of deletion DI species del 2.5 and del 3.3 were not detectable by RNase protection 3 days p.i., but could be clearly demonstrated 24 h p.i., using a specific RT-PCR assay (Fig. 7B). In each panel, the signal specific for the considered DI species (arrow) was present in cells infected with Pi25.C2 virions (lane 3), but not with mock- (lane 1) or P0-infected cells (lane 2). UV-irradiation resulted in the disappearance of the specific signal (lane 5), but replication was restored by rescue of the UV-irradiated defective stock with P0 standard stock virus (lane 6). These results indicated that such DI particles could be used as natural minireplicons to assay Ebola standard virus support functions.

**DISCUSSION**

Experiments involving live Ebola or Marburg virus have been limited by the need for a maximum biosafety laboratory (BSL4) containment laboratory, thus hindering investigation of classical phenomena such as virus interference or attenuation. Aberrant forms of Ebola Sudan virus (Ellis et al., 1979) and other filoviruses (Geisbert et al., 1995) described earlier, may have been the first morphological evidence of filovirus DI particles. The current study focused on the Zaire subtype of Ebola virus due to its recent reemergence in Africa (Georges-Courbot et al., 1997) and high lethality of the human infection (Bwaka et al., 1999). The data presented here show that Ebola virus DI particles could be generated and virus persistently infected cell lines established using the classical approach of undilute virus passage. These DI particles included both classic deletion and copy-back forms, which appeared to be produced with comparable efficiency during initial passages. The population of virus DI particles evolved during the virus and persistently infected cell passage series, with shorter defective RNAs outcompeting longer forms, and a progression from the detection of both deletion and copy-back defective RNAs in early material to detection of only copy-back defective RNAs in the later passages. Such features are consistent with earlier data on DI particles of rhabdovirus and
paramyxoviruses, which suggest that shorter DI particles replicate and interfere more efficiently than longer DI particles and that possession of the presumed higher efficiency antigenomic promoter on the 3’ termini of both genome and antigenome copy-back DI RNAs affords them a replicative advantage over deletion DI (or standard viruses), which possess the lower efficiency genomic promoter on their genome RNAs (Re, 1991; Calain and Roux, 1995). Based on the pattern of competition of Ebola DI RNAs seen here, it would appear that similar differences in efficiency in genomic and antigenomic promoters exist for the filoviruses and paramyxoviruses, which are phylogenetically more closely related to each other than they are to other virus families (Morzunov et al., 1995).

The detailed characterization of the naturally occurring Ebola virus minireplicons described here, has implications for the approaches to the development of reverse genetics systems for Ebola virus. As these minireplicons undergo efficient replication and packaging into infectious particles they must contain the necessary genome elements for RNA encapsidation and polymerase complex binding and function. Possession of competent promoters at the 3’ termini of DI genome and antigenome RNAs is an obvious requirement, but other more subtle features of an efficient replicon may also be contained. For instance, a relative need for any replicative molecule to have a genome length of a multiple of six nucleotides (frequently referred to as the “rule of six”), has been formally demonstrated for several members of the subfamily Paramyxovirinae, including Sendai (Calain and Roux, 1993), human parainfluenza 3 (Durbin et al., 1997), and simian virus 5 (Murphy and Parks, 1997), and is also likely to apply to measles virus (Sidhu et al., 1995). However, replication of minigenomes of respiratory syncytial virus, the prototype of the subfamily Pneumovirinae, were not constrained by a rule of six or any other common integer divisor (Samal and Collins, 1996). Virus mRNA editing is carried out by viruses of the subfamily Paramyxovirinae, but not those of the subfamily Pneumovirinae. As the presence of a cotranscriptional editing site in the P gene results in instability at this site during genome replication (Hausmann et al., 1996), it seems possible that the rule of six in Paramyxovirinae subfamily viruses may be evolutionarily linked to their need to eliminate genomes with length variations due to RNA editing. The possibility of a length rule applying in Ebola virus replication was considered due to the phylogenetic relatedness of Ebola virus to the paramyxoviruses and the RNA editing seen during Ebola virus glycoprotein mRNA synthesis (Sanchez et al., 1996; Volchkov et al., 1995). The recently revised genome length (18960 nucleotides; Towner and Sanchez, unpublished; Genbank Accession Nos. L11365 and X67110; Sanchez et al., 1993; Volchkov et al., 1999) of the Ebola Zaire virus, Mayinga strain, is divisible by six. However, only one of the seven Ebola virus naturally occurring minireplicons characterized here were found to be divisible by six. Further analysis revealed no other common integer divisor among these Ebola minireplicons (Fig. 5). Although Marburg virus does not carry out RNA editing, the genome of the Musoke strain (19104 nucleotides; Feldmann et al., 1992; revised in GenBank Z12132) is divisible by six, but not that of the Popp strain (19112 nucleotides; Bukreyev et al., 1995). Based on these observations it appears that a length rule will not be applicable to the filoviruses.

The apparent lack of an integer length rule is one more feature shared by the viruses of the Filoviridae family, respiratory syncytial virus (RSV), and other members of the Pneumovirinae subfamily. RSV is also phylogenetically closer to the filoviruses than are other viruses (Morzunov et al., 1995), and gene overlaps exist in RSV and Ebola and Marburg viruses (Collins, 1991; Sanchez et al., 1993). In addition, their attachment glycoprotein is O-glycosylated (Feldmann et al., 1994), and their glycoprotein genes encode both membrane-anchored and soluble forms of the glycoprotein, albeit by different mechanisms (Sanchez et al., 1996; Roberts et al., 1994). Given the similarities, results of RSV studies may provide insights applicable to filovirus biology and approaches to antiviral and prototype vaccine development.

The extent of the Ebola virus RNA terminal sequences necessary for efficient Ebola virus replication and packaging is currently unclear due to the lack of detailed mapping experiments and the unusually long terminal noncoding regions. The Ebola Zaire virus genome 3′ terminus contains a 469-nt region upstream of the N protein open reading frame (Sanchez et al., 1989), with the terminal 53 nt up to the N mRNA initiation site making up the leader region (Sanchez et al., 1993). The position of the L mRNA transcription termination-polyadenylation site has recently been mapped to genome region UAAAUUUUUU at genome nucleotide positions 18273 to 18283 (position as corrected by Towner and Sanchez, unpublished), resulting in a 677-nt trailer region at the genome 5′ terminus (Volchkov et al., 1999). It is reasonable to assume that the shortest Ebola virus minireplicons described here (del 3.3 and cb6.2; see Fig. 4) carry all the cis-acting sequences necessary for efficient virus replication and packaging since these minireplicons were shown to propagate from cell to cell through virus particles. From these structures, we can infer that 155 nt of the genomic 3′ terminus and 176 nt of the antigenomic terminus are sufficient to allow binding of the viral polymerase complex and subsequent initiation of RNA replication and encapsidation. Recently, a Marburg virus artificial minigenome has been successfully replicated which contained 106 and 439 nt of the standard virus genome 3′ and 5′ termini, respectively, flanking a CAT reporter gene (Muhlberger et al., 1998). More recently, this same group has described the successful replication of an Ebola minireplicon containing 472-nt leader
and 731-nt trailer regions flanking the CAT reporter (Muhlberger et al., 1999). Together these data will aid the design and manipulation of Ebola virus artificial mini-genomes for use in development of Ebola virus reverse genetics systems.

The natural reservoir of Ebola virus remains an enigma. Some filovirus infections of humans have been initiated by contact with sick or dead monkeys or apes, or their tissues (Georges-Courbot et al., 1970; LeGuennou et al., 1995), but most cases have occurred through close contact with disease patients or their fluids (World Health Organization, 1978a,b; Dowell et al., 1999). Insect or plant hosts are not considered likely based on the lack of virus growth in insect cells and the absence of any sequence identity with known plant RNA viruses (Van der Groen et al., 1978). The most significant clue is likely the high sequence identity seen between an immunosuppressive domain present in envelope glycoproteins of retroviruses of higher vertebrates and a similar domain in filovirus GP proteins (Volchkov et al., 1992; Sanchez et al., 1993; Lu et al., 1995; Gallaher, 1996). Such data have suggested mammals or birds as potential hosts although Ebola virus is generally considered to be an acute infection resulting in extensive cytopathology in many mammalian cells. However, the ease with which we were able to establish an Ebola virus persistently infected mammalian cell line and resulting continuous shedding of infectious virus, along with recent data on experimental infection of bats (Swanepoel et al., 1996), suggest that mammal or bird potential hosts of Ebola virus warrant further investigation.

Filovirus persistence following naturally acquired infection of humans has been reported previously. Marburg virus was isolated from the seminal fluid and anterior chamber of the eye of convalescent hemorrhagic fever patients up to 80 days post-onset of disease (Martin, 1969; Smith et al., 1982). Ebola Sudan virus has been recovered from the seminal fluid of a convalescent patient 61 days post-onset of disease (Emond et al., 1977). More recently, Ebola Zaire virus RNA could be detected for up to 33 days after disease onset in vaginal, rectal, and conjunctival swabs of a patient, and up to 101 days after disease onset in the seminal fluid of 4 patients (Rodriguez et al., 1999). Infectious virus was detected in the seminal fluid of one of these patients 82 days after disease onset. Our demonstration that a cell line of primate origin can be persistently infected with a filovirus adds credence that such instances of prolonged shedding in humans are genuine cases of persistent infection in vivo.

Ebola live-attenuated vaccines could be of some value, particularly an Ebola Reston vaccine for use in animals in primate-holding facilities (Rollin et al., 1999). Our study suggests that the production of an Ebola virus attenuated strain might be possible using the viruses generated by undilute passage or from persistently infected cells. In at least two examples, DI particles were detected in live-attenuated vaccines and were suggested to play a role in attenuation (Calain and Roux, 1988; McLaren and Holland, 1974; Roux et al., 1991). Along these lines, it will be of interest to determine the virulence in experimental animals of the Ebola viruses released from the virus undilute passage or persistently infected cell series. In addition, reverse genetics techniques offer the prospect of producing pure, nonvirulent, immunogenic Ebola viruses by engineering of a cloned infectious virus genome.

**MATERIALS AND METHODS**

**Cells and virus passages**

All experiments involving infectious Ebola viruses were carried out in a biosafety level 4 (BSL4) laboratory. Vero E6 (ATCC CRL-1586) cells were used for the propagation of Ebola virus and for the generation of virus DI particles. A Vero ATCC CCl-81 cell line was used for rescue of purified defective virus stocks because of their tolerance to repeated transfections and vaccinia infections which were anticipated in future reverse genetic studies. Prior to initiation of virus undilution passage experiments, several stocks of Ebola-Zaire, Mayinga strain virus (originally isolated from a 1976 patient) were assessed for their ability to grow rapidly and produce high cytopathology. A plaque purified virus stock (8066567) had been prepared earlier from the patient sample by 3 passages in Vero cells followed by 3 plaque purifications and selection of large plaques (Sanchez et al., 1996). This virus was found to grow more quickly (causing extensive CPE by 3 days postinfection) than earlier non-plaque purified virus stocks or other nonmutant plaque purified viruses. Due to these growth capabilities, this virus stock was chosen to initiate the serial undiluted passage of Ebola virus in Vero E6 cells. Previous nucleotide sequence analysis of virion RNA from this stock showed that an additional U base has been inserted in a string of 7 Us representing the glycoprotein gene editing site, resulting in the synthesis of a full-length glycoprotein from primary, unedited transcripts (Sanchez et al., 1996). A virus working stock was made from the clarified supernatant of cells infected with stock 8066567 at an m.o.i. of approximately 1. It is worth noting that the 8-U genotype of these viruses did not vary during the virus undilution passage series or persistent infection passages described here (data not shown).

Preliminary experiments also indicated that during tissue culture cell infection, release of Ebola virus infectious particles into cell culture medium was incomplete with significant amounts of infectious virus remaining cell-associated. In addition, earlier Ebola virus infectivity and electron microscopy studies had shown that heterogeneous elongated virus particles could be resolved into
smaller more homogeneous sized particles with increased infectivity by sonication (Russ Regnery, personal communication). For these reasons, undilute virus passage experiments were carried out using sonicates of harvested infected cells suspended in their supernatant fluid, in order to maximize virus yields.

Serial passages were initiated by infecting cells with working stock virus at an m.o.i. of 0.2. Extensive CPE was evident after 5 days and cells were scraped in approximately 10 ml of their supernatant per 75-cm² flask. The cell suspension was sonicated for 60° on a refrigerated water bath (Tekmar7 Sonic Disruptor, position 8, 90% duty cycle). The resulting virion preparation was called “P0” and used undiluted to initiate the next infection at a high m.o.i. Subsequent serial passages (from “P1” to “P10”) were carried in the same way, using the undiluted sonicate of the previous infection as inoculum. At each passage, one T75 flask was harvested for analysis of total RNA, and another two T75 flasks for preparation of the next inoculum. In some experiments (Fig. 4), additional controls used RNA from Ebola virus stock 806346. This stock was generated independently from stock 806567 by serial passages of the Mayinga strain at low m.o.i. in Vero cells without plaque-purification. Plaque titrations were carried out according to standard techniques (Ksiazek et al., 1999).

Passage, immunofluorescence, and storage of Ebola persistently infected cells

Ebola virus persistently infected cells were split 1:4 at intervals between 7 and 14 days, using trypsin-EDTA solution (GIBCO BRL). Rapid assessment of continuing infection was carried out by immunofluorescence assay essentially as described earlier (Ksiazek et al., 1999). Stocks of persistently infected cells were made in 10% DMSO-containing medium and stored in liquid nitrogen.

Interference and superinfection assays

For assay of interference, T25 flasks of confluent Vero E6 cells were infected with a mixture of virus stock (m.o.i. of 0.3) and 1 ml of test preparation. Controls included identical concentrations of stock alone, test preparation alone, and medium alone. Cells were checked daily for appearance and intensity of CPE. Virions released were measured 3 days p.i. by plaque assay of the cell supernatants. Superinfection assays were carried out using T25s of newly confluent Ebola persistently infected cells, as well as normal E6 cells, either mock infected or superinfected with stock P0 virus at an m.o.i. of 0.1 for 1 h. Fresh medium was then added and sampled 2 and 5 days p.i. by plaque assay for virus release.

Ebola virus riboprobes

Plasmid pEBO 7 was constructed by inserting a PCR fragment spanning nucleotides 17343 to 18960 of the Ebola Zaire virus Mayinga strain, into pCR II (Invitrogen) cloning vector. A positive riboprobe (rL) extending to the 5’-genomic terminus was obtained after linearization with Mlu1 and transcription with T7 RNA polymerase. Plasmid pGEM-EV3 has been described previously (Sanchez et al., 1993). After digestion with NcoI, a positive riboprobe (rVP40) including 1014 nucleotides of the VP40 gene, was produced by transcription with SP6 RNA polymerase. Plasmid pEBO 47 (kindly provided by A. Sanchez) was digested with SalI, followed by transcription with SP6 polymerase to produce a positive riboprobe (rNP) spanning the first 469 nucleotides of the 3’-genomic terminus. For the detection of defective molecules cb 6.2 (see Results) by RNase protection, plasmid pEBO 43 was constructed by inserting into pSP73, an RT-PCR product spanning the breakpoint region of the copy-back molecule (for orientation, see Fig. 1). After linearization with EcoRV and transcription with T7 RNA polymerase, the resulting riboprobe contained the entire inverted complementary repeat (negative sense) followed by 106 nucleotides of nonrepeat sequences (positive sense). Additional RNA flanking sequences were transcribed from polylinker regions.

For Northern-blot analysis, riboprobes were made in 20-µl reactions containing 1 µg of linearized plasmid, 40 units of RNase inhibitor (Boehringer), 1 mM each of cold CTP, GTP, and ATP, 0.05 mM of cold UTP, 50 µCi of [α-32P]UTP (Amersham), and 20 units of the appropriate RNA polymerase (SP6 or T7 polymerase, Boehringer) in buffer according to the manufacturer’s instructions. For RNase protection experiments (6–12 samples analyzed), the above reaction was scaled up fivefold and the riboprobe was purified on a G-50 Sephadex column.

Northern blot

Total cell RNA was extracted and purified using a commercially available kit (RNaid, Bio101 Inc.). One microgram of each RNA sample was electrophoresed on a 1.2% agarose formaldehyde denaturing gel, transferred to nitrocellulose according to standard procedure (Sambrook et al., 1989, pp. 7.43–7.48). Cold transcripts of opposite polarity to the rL riboprobe were run in parallel as size markers. The appropriate riboprobe was then added and hybridization was carried out at 48°C for a minimum of 8 h. Blots were analyzed by exposing nitrocellulose filter to X-ray film.

Ribonuclease protection

Total cell lysates were directly probed for defective molecules using the Lysate Ribonuclease kit as described by the manufacturer (United States Biochemical). The overnight hybridization temperature was 45°C. As a control, the riboprobe was incubated in cell lysate buffer, and treated as the test samples, except the ribonuclease
mixture was omitted. Protected fragments were analyzed on a 4% acrylamide-urea gel.

RT-PCR and nucleotide sequence analysis

RNA was extracted from cells or purified virions as described for Northern blot analysis. Reverse transcription of 1 µg RNA was carried out with Superscript II (GibcoBRL) according to manufacturer’s instructions. The resulting cDNA preparation was diluted fivefold with water and 2 µl was used for PCR amplification using Expand High Fidelity PCR System (Boehringer) as indicated in the manufacturer’s protocol. The program used for amplification of defective species in Fig. 1 was: 94°C for 2 min, followed by 15 cycles of [94°C for 15 s/45°C for 30 s/72°C for 3 min] followed by 20 cycles of [94°C for 15 s/45°C for 30 s/72°C for 3 min with 20 s additional elongation in each cycle] followed by 3 min at 72°C. PCR products were analyzed on 3% agarose gels and purified using the Sephaglas BandPrep kit (Pharmacia). Purified PCR products were directly sequenced, or cloned (pCR2.1 cloning kit, Invitrogen) when direct sequencing was problematic. Inserts of six independent clones were confirmed to be identical by restriction analysis before sequences were determined using original PCR primers, as well as vector primers, by the dyedideoxy-termination method (ABI Prism Dye Terminator FS, Perkin-Elmer).

Purification and UV-irradiation of virions

Culture supernatants were clarified by low speed centrifugation (Beckman GPR centrifuge, 2500 rpm for 5 min), then layered on a 10-ml cushion of 20% sucrose and centrifuged for 20 min at 60,000g in a SW28 rotor. Virion pellets were resuspended in DMEM and stored in liquid nitrogen. When required, standard virus particles were eliminated from DI virion preparations by UV irradiation. Samples were put in 6-well plates (1 ml/well) and irradiated in an ultraviolet crosslinker (Model UCV-508, Ultra-lum, Inc.). Doses of 5 ml/cm² were empirically determined to cause a steady decrease of 100-fold in the virus titer of Ebola Zaire virus. In rescue experiments, virion preparations from persistently infected cells were irradiated with 20 mJ/cm² to determine if the 2 µl used for for PCR amplification using Expand High Fidelity PCR System (Boehringer) as indicated in the manufacturer’s protocol. The program used for amplification of defective species in Fig. 1 was: 94°C for 2 min, followed by 15 cycles of [94°C for 15 s/45°C for 30 s/72°C for 3 min] followed by 20 cycles of [94°C for 15 s/45°C for 30 s/72°C for 3 min with 20 s additional elongation in each cycle] followed by 3 min at 72°C. PCR products were analyzed on 3% agarose gels and purified using the Sephaglas BandPrep kit (Pharmacia). Purified PCR products were directly sequenced, or cloned (pCR2.1 cloning kit, Invitrogen) when direct sequencing was problematic. Inserts of six independent clones were confirmed to be identical by restriction analysis before sequences were determined using original PCR primers, as well as vector primers, by the dyedideoxy-termination method (ABI Prism Dye Terminator FS, Perkin-Elmer).

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REFERENCES


