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Rapid Communication

Generation of eGFP expressing recombinant Zaire ebolavirus for analysis of early pathogenesis events and high-throughput antiviral drug screening

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Abstract

Zaire ebolavirus causes large outbreaks of severe and usually fatal hemorrhagic disease in humans for which there is no effective treatment or cure. To facilitate examination of early critical events in viral pathogenesis and to identify antiviral compounds, a recombinant Zaire ebolavirus was engineered to express a foreign protein, eGFP, to provide a rapid and sensitive means to monitor virus replication in infected cells. This genetically engineered virus represents the first insertion of a foreign gene into ebolavirus. We show that Ebola-eGFP virus (EboZ-eGFP) infects known early targets of human infections and serves as an ideal model to screen antiviral compounds in less time than any previously published assay.
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Keywords: Zaire ebolavirus; Hemorrhagic disease; eGFP; Recombinant virus

Introduction

The emerging viral pathogens Ebola virus (EboV) and Marburg virus (MbgV) (family *Filoviridae*) are known to cause devastating disease in human and non-human primates. In humans, signs and symptoms of Ebola hemorrhagic fever (EHF) can include gastrointestinal (nausea, abdominal pain), respiratory (shortness of breath, cough) vascular (mucosal hemorrhages, visceral hemorrhagic effusions, coagulopathy), and central nervous system (headache, dementia, coma) manifestations (Sanchez et al., 2001). The case fatality ranges between 50% and 90% for some species of EboV with death typically occurring quickly, within 7 to 10 days after onset of symptoms. Due to the severity of the disease, the rapid onset of symptoms, and the ease of human to human transmission, these viruses are studied exclusively under biosafety level 4 (BSL-4) containment and are listed on the Centers for Disease Control and Prevention (CDC) Category A list of potential bioterrorist agents. Early viral amplification is thought to occur in mononuclear phagocytes and dendritic cells, followed by massive liver and spleen infection. In some cases there is significant endothelial cell infection and leakage (hemorrhage) in addition to blood coagulation abnormalities such as disseminated intravascular coagulation (Geisbert et al., 2003; Sanchez et al., 2001; Zaki and Goldsmith, 1999). Unfortunately, the mechanisms underlying the severe pathogenesis are only partially understood, highlighting the need to further understand viral replication, especially the early critical events in an infection, as well as develop effective antiviral therapies. Currently, there is no effective antiviral therapy for humans infected with either EboV or MbgV, although there have been a number of

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compounds shown to exhibit specific antiviral properties in tissue culture infections and animal models. One class of inhibitors specifically targets virus entry by interfering with proposed interactions between the highly glycosylated viral glycoprotein (Barrientos et al., 2003) and the cellular C-type lectin adhesion molecules DC-SIGN and L-SIGN. Treatment of cultured cells with either of two compounds in this class, cyanovirin-N (CV-N) or BH30sucMan, result in a dramatic inhibition of virus entry (Barrientos et al., 2003; Lasala et al., 2003). Overall, however, the success of these two drugs is limited. When CV-N is used to treat EboV-infected mice, death is delayed but not prevented (Barrientos et al., 2003). Complete protection of mice is achieved using a different type of inhibitor, S-adenosylhomocysteine hydrolase (SAH) (Bray et al., 1999; Huggins et al., 1999) although it is effective only when mice are treated the day before or the day of virus challenge. The antiviral effect of SAH inhibitors was first thought to be due to reduced methylation of the 5′ cap of viral messages, but Bray et al. (2002) now believe the antiviral properties may be attributed to an increase in IFN-α production, consistent with the protective role of the innate immune response in the mouse model of EboV infection (Mahanty et al., 2003). To date, these compounds have not shown efficacy in non-human primate models. More recently, using a non-human primate model, Geisbert et al. (2003) have demonstrated a partial amelioration of EHF in rhesus monkeys using recombinant nematode anticoagulant protein C2 (rNAPc2), a potent inhibitor of tissue factor-initiated blood coagulation. This treatment regime increased survival from 6% to 33% of treated animals. One of the major stumbling blocks towards the goal of developing more effective therapies has been the lack of a sensitive high-throughput assay to measure the effects of potential antiviral compounds. The assays currently in use for such purposes take a week (Barrientos et al., 2003; Huggins et al., 1999) and only measure virus replication indirectly by monitoring cytopathic effect (CPE) or cellular neutral red uptake. In this study, we have used reverse genetics to generate a full-length cDNA clone of Zaire ebolavirus into which we inserted a foreign reporter gene, eGFP. Although eGFP has been successfully inserted into genomes of other (smaller) single-strand negative sense RNA viruses, EboV is almost 19 kb in length and we were uncertain if the genome size would create constraints for insertion of foreign genes. However, virus rescue from this clone (EboZ-eGFP virus) was successful, and we found that it grows similarly to that of wild-type EboV yet causes infected cells to fluoresce brightly. EboZ-eGFP virus thus provides a rapid, sensitive, and quantitative readout of virus replication and dissemination in infected cells that will allow examination of early pathogenic events and high-throughput drug screening in less time than any previously described assay. In addition, this is the first description of an insertion of a foreign gene of any kind into a member of the family *Filoviridae*.

Results

Generation of infectious clone and virus rescue

We successfully generated and rescued virus from a full-length clone of Zaire ebolavirus (Mayinga strain) into which we cloned a *Bsi*W1 restriction site present in the NP 3′ UTR (Fig. 1). This “wild-type like” virus was termed EboZ-*Bsi*W1. The *Bsi*W1 restriction site was then used to insert the eGFP open reading frame preceded by a 90-nucleotide fragment containing an authentic transcription stop sequence, to direct the termination of the NP mRNA, and a transcription start sequence to direct the initiation of the eGFP message. Compared to rescue of non-eGFP expressing EboZ-*Bsi*W1 virus, monitoring virus rescue from the pEbola-eGFP clone was simple and rapid. Within 48–72 h after transfection, usually 5–15 cells per well of a 6-well dish would fluoresce brightly. If we used CPE to monitor virus rescue, for either EboZ-eGFP or EboZ-*Bsi*W1, we

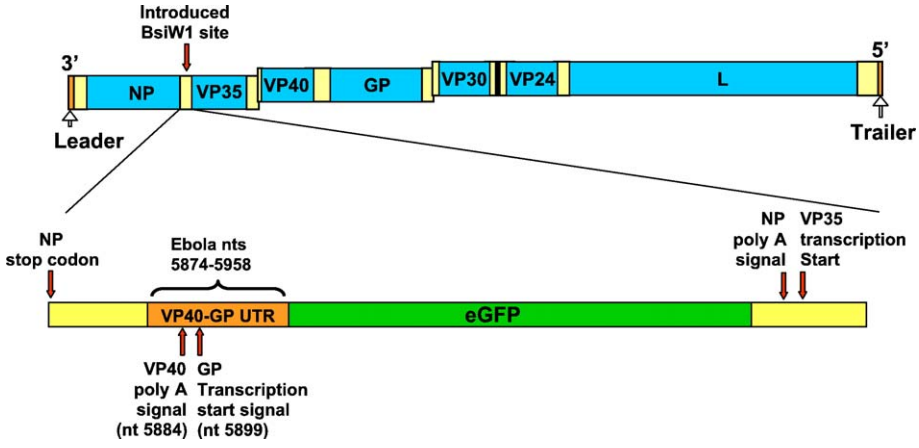


Fig. 1. The schematic diagram depicts eGFP insertion into the full-length Zaire ebolavirus ’76 Mayinga clone at the introduced *Bsi*W1 restriction site in the NP 3′ UTR. Diagram also shows the approximate location of the inserted transcriptional stop and start signals to facilitate NP and eGFP expression.

typically had to wait 5–10 days after passage of the transfected cell supernatant onto uninfected Vero E6 monolayers, consistent with the results of others (Neumann et al., 2002).

Characterization of EboZ-eGFP growth properties in Vero E6 cells

In order to determine the utility of eGFP expression as an effective reporter for virus infection, Vero E6 cells were infected at a multiplicity of infection (MOI) of two and monitored at various times post-infection for eGFP fluorescence. As shown in Fig. 2A, the expression of eGFP in infected cells is both rapid and robust, thus allowing simple discrimination of infected cells within 24 h. Similarly, virus plaques are also easy to identify and quantitate in the absence of any immuno- or neutral red staining (Fig. 2B).

Because the eGFP ORF was inserted immediately after the NP gene all downstream viral genes might be expected to be downregulated due to the sequential and polarized gene expression characteristic of single-strand negative sense RNA viruses. For this reason, the growth properties of EboZ-eGFP could be attenuated. In order to examine this possibility, a single cycle growth analysis of EboZ-eGFP was performed side-by-side with EboZ-*Bsi*W1 virus as well as wild-type Mayinga '76 Zaire ebolavirus (EboZ-wt). As shown in Fig. 3A, growth analysis reveals that EboZ-eGFP growth kinetics are

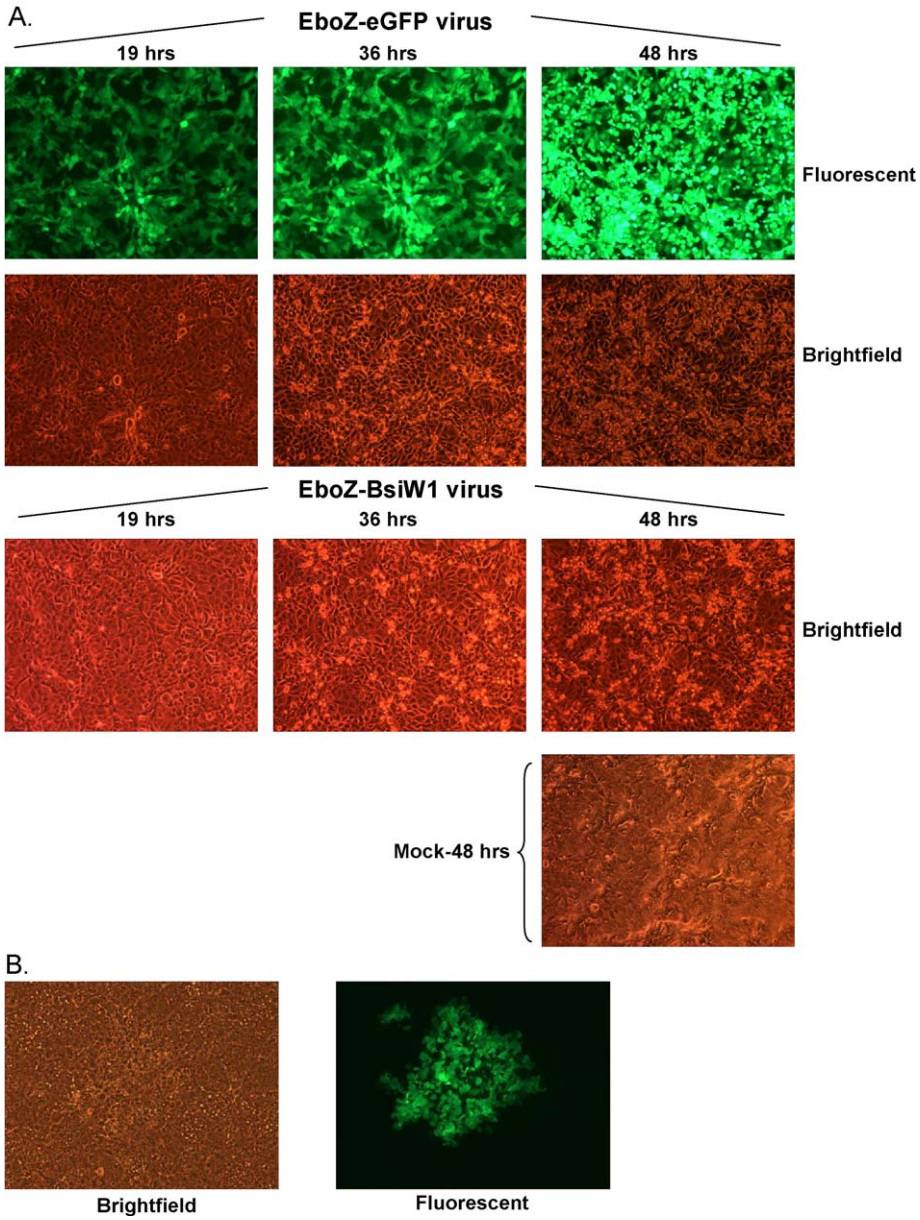


Fig. 2. Demonstration of eGFP fluorescence in infected Vero E6 cells. (A) EboZ-eGFP and EboZ-*Bsi*W1 infections at 18, 32, and 48 h post-infection monitored using either fluorescent or brightfield microscopy. (B) Brightfield and fluorescent microscopy of a day 7 plaque of EboZ-eGFP immobilized under 1% agar.

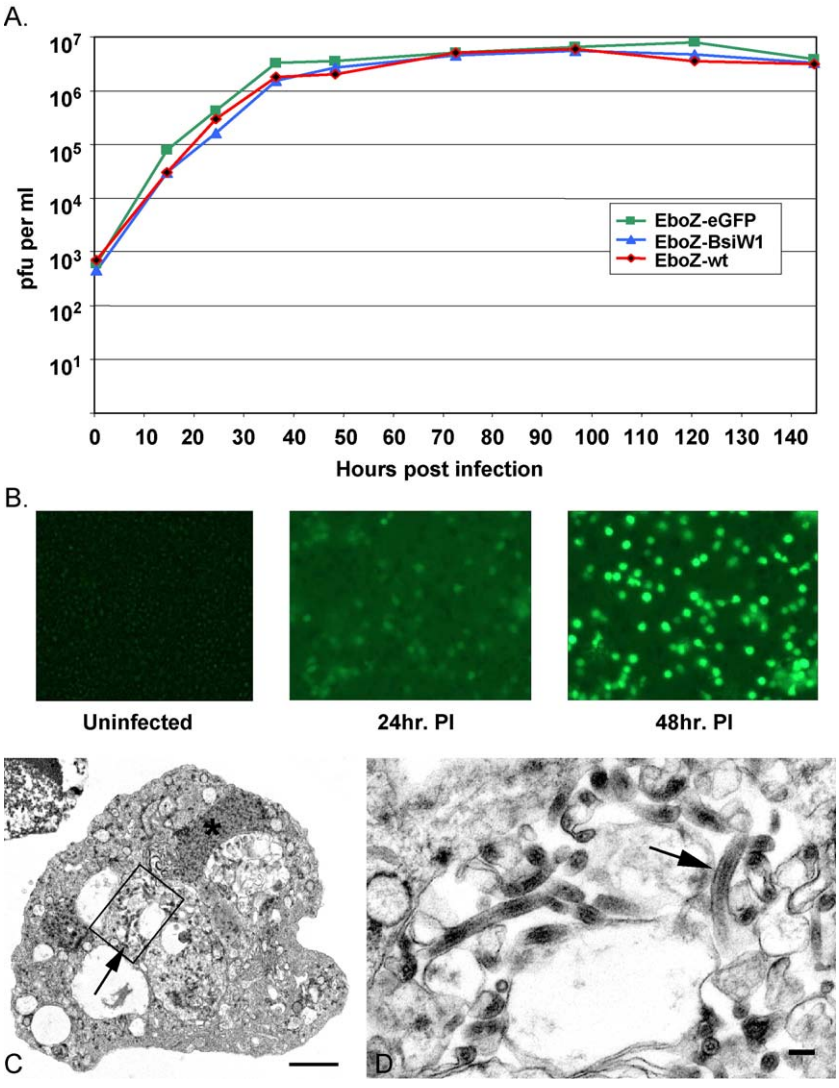


Fig. 3. (A) Time course of Vero E6 cells infected with EboZ-eGFP, EboZ-*Bsi*W1, or EboZ-wt. Vero E6 cells were infected at a MOI of two and virus titers in the supernatants were determined at the indicated times post-infection. (B) Fluorescent microscopy of human PBMCs either mock-infected or infected with EboZ-eGFP and analyzed at the indicated times post-infection. (C) Electron micrograph of a human macrophage infected with EboZ-eGFP virus. (*, nucleocapsid inclusion. Scale bar, 1 μm). (D) At a higher magnification of area pointed to (boxed) in (C), ebolavirus particles are clearly visible (arrow) (Scale bar, 100 nm).

essentially identical to that seen with either EboZ-wt or EboZ-*Bsi*W1. Consistent with the single cycle growth analysis, the plaque morphology of EboZ-eGFP was indistinguishable from that of either of the other two viruses tested (data not shown). In addition, we studied the genetic stability of eGFP expression by undiluted passaging of the virus multiple times in Vero E6 cells. After 25 passages with no major diminuation of eGFP signal in infected cells (data not shown), it was concluded that the eGFP insertion was stable. Some slight variations in eGFP intensity were observed from one passage to the next. However, these variations were not consistent and were likely the result of replication interference by defective interfering particles accumulated during undiluted virus passage as has been previously demonstrated for EboV (Calain et al., 1999).

EboZ-eGFP infection of human PBMCs

One of the primary goals for inserting eGFP into EboV was to generate a virus with growth and tropism properties similar to that of wild-type virus, but whose replication would be more easily and rapidly visualized. Early critical events in human and animal infections are largely unknown due to the difficulty in detection of cells infected soon after exposure. A virus such as EboZ-eGFP could be useful for demonstrating primary sites of replication in animal model infections. Previous studies have identified monocytes and macrophages as primary targets for filovirus infection in human and animal models (Connally et al., 1999; Feldmann et al., 1996; Geisbert et al., 2003; Gupta et al., 2001; Jaax et al., 1996; Stroher et al., 2001; Zaki and Goldsmith, 1999). Therefore, to further investigate if EboZ-eGFP virus infects

the same known early targets of wild-type Zaire ebolavirus, human PBMCs isolated from healthy volunteers were infected in vitro with 1 MOI of EboZ-eGFP. The results of this analysis are shown in Fig. 3B in which rapid green fluorescence of adherent PBMCs is detected within 24 h post-infection. Subsequent FACS analysis revealed that within 24–48 h, approximately 30% of class II+ cells were GFP+, and of these class II+ GFP+ cells, none were B cells by CD19 co-staining (data not shown). As it is well established that HLA class II+ cells consist primarily of B cells, dendritic cells, and macrophages, these data indicate that macrophage and dentritic cells are the likely viral targets within the PBMC population. To re-confirm the size of the macrophage population within class II+ cells, we stained class II+ cells for the macrophage marker CD14 and found that approximately 30% cells were CD14+. Further electron microscopy analysis of PBMCs infected with EboZ-eGFP confirm that macrophages are infected. As shown in Fig. 3C and D, EboZ-eGFP virus production in human macrophages is evidenced by the presence of viral nucleocapsid inclusions and the generation of mature viral particles.

A rapid assay for screening antiviral compounds

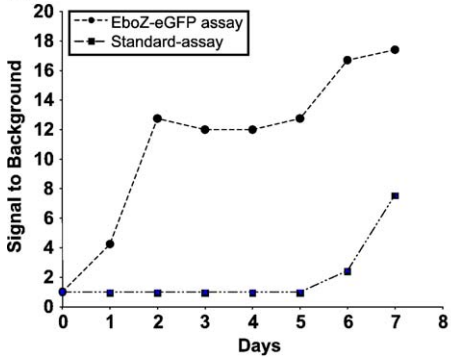
As a proof of principle for using the EboZ-eGFP virus to rapidly screen antiviral compounds, consensus interferon alpha (conIFN-α), a compound with known EboV antiviral properties, was used at multiple concentrations to treat Vero E6 cells infected with either EboZ-eGFP virus or a control Zaire ebolavirus. The infected cells were monitored for evidence of virus replication by either fluorescence microscopy (for EboZ-eGFP virus) or a previously published CPE-based assay (Huggins et al., 1999). Using a logit4 computer algorithm as previously described (Huggins et al., 1999; Paragas et al., 2004), the IC50 values were determined on days 1 and 2 using the EboZ-eGFP assay and on day 7 using the CPE-based assay. The signals to background level for each assay are presented in a graph shown (Fig. 4A) along with the calculated IC50 values determined by each method (Fig. 4B). For the EboZ-eGFP assay, the data show an overall signal to background ratio of approximately 12:1 within the first 48 h, ultimately reaching a level of 16:1. By contrast, the CPE-based assay requires 7 days to reach a stage where the effect of virus replication can be measured, a point at which the signal to background ratio is approximately half of that obtained using the EboZ-eGFP assay.

Discussion

In this study, we report the first insertion of a foreign gene, eGFP, into Zaire ebolavirus. This was achieved by first generating a full-length clone of Zaire ebolavirus, strain Mayinga, and inserting eGFP as an extra transcription unit in between the NP and VP35 genes. These results show that the initial concern about size limitations for insertion of a foreign gene into an already large (approximately 19 kb) genome were unfounded. Insertion of eGFP into EboV extended the upper size limit of filovirus genomes to 19,773 nucleotides. Surprisingly, single-step growth analysis in cell culture revealed that the recombinant virus showed no detectable attenuation in either the rate of growth or the ultimate titers reached. The reporter gene expression is stable over many passages, consistent with previous reports of foreign gene insertions in other single-strand negative sense viruses (Duprex et al., 1999). Infection of human PBMCs demonstrated that EboZ-eGFP virus infects macrophages, an early target in human infections. Furthermore, we showed that infection of human macrophages is easily detectable within 24 h due to the robust eGFP fluorescence present in infected cells.

After establishing that EboZ-eGFP virus behaves in a manner similar to that of wild-type Zaire ebolavirus in tissue culture and in vitro human PBMC infections, we demonstrated in proof of principle that this recombinant virus serves as an effective tool to screen compounds for antiviral properties. The data presented clearly demonstrate that the ability to monitor the progression of an ebolavirus infection is greatly enhanced by the generation of eGFP as a reporter of virus replication. This robust signal allowed the calculation of consistent IC50 values in less than a quarter

A.



B.

Virus	IC50	Day
EboZ-Mouse Adapted	0.257 μg/ml	7
EboZ-eGFP	0.133 μg/ml	1
EboZ-eGFP	0.122 μg/ml	2

Fig. 4. (A) A time-dependent line graph of the signal to background ratio of the CPE-based and the eGFP fluorescent-based drug discovery assays. The axes are signal to background versus time.

of the time of previously published assays. The more rapid readout afforded by the EboZ-eGFP virus is an important feature, not only to reduce the overall time needed for screening compounds on a high throughput basis, but also to measure viral inhibition of compounds with short half-lives. Many potential drugs will degrade and/or become inactive well before the 7 days required for completion of the CPE-based assay, thus allowing for variable levels of virus recovery during the period between drug inactivation and the time of neutral red uptake. This in turn could lead to a less accurate calculation of the IC50 values. An additional attribute of the EboZ-eGFP virus is that, compared to the CPE-based assay, eGFP fluorescence is directly indicative of virus replication because it is a gene product wholly dependent, and thus proportional to, viral RNA replication and transcription. CPE on the other hand is a product of multiple downstream cellular events brought about by the action and accumulation of viral gene products, such as the glycoprotein, and is therefore much delayed in appearance compared to virus replication itself.

We anticipate EboZ-eGFP virus will also be of great value with pathogenesis studies, particularly those focused on critical early events in animal-model infections including non-human primates. The natural reservoir for Zaire ebolavirus, or any filovirus for that matter, remains to be elucidated. EboZ-eGFP virus could be very useful for detecting horizontal and/or vertical transmission following experimental infections of candidate reservoir species. The fluorescence of the eGFP signal should be more robust to that observed by standard immunohistochemistry (IHC), as has been shown for eGFP-expressing measles virus in infected astrocytoma cells (Duprex et al., 1999), and should therefore complement standard IHC methods. Secondly, sample preparation and analysis should be considerably faster and easier because tissue fixation and antibody incubations will not necessarily be required.

Material and methods

Generation of the full length clone, insertion of eGFP and virus rescue

The full-length clone was generated from multiple cDNA fragments derived from total RNA isolated from cells infected with the 1976 Mayinga strain of Zaire ebolavirus. The cDNA fragments were ligated together using conventional cloning techniques to form three subgenomic clones roughly corresponding to NP-GP, GP-L, and L. The three subgenomic clones were then sequentially ligated together to form a full-length positive sense clone under the control of a phage T7 promoter followed by a single non-viral G residue. The L sequence was followed by a hepatitis delta ribozyme and T7 transcription terminator. The sequence of the full-length clone was compared to a previously determined sequence for the ’76 Mayinga strain of Zaire ebolavirus which is identical to that listed by Volchkov et al. (GenBank accession numberNC002549). In total, there were seven nucleotide changes in addition to the three nucleotide insertions used to generate the *Bsi*W1 restriction site. All but one of the seven changes were in non-coding sequences and were left in the full length clone to serve as genetic markers. The changes were G to C at nt 2833, A to G at nt 3803, A to G at nt 4187, T to C at nt 11193, G to A at nt 14187, and a T deletion at nt 18614. The single G to A mutation at position 18138 conferred an M to I amino acid change near the C-terminus of L. This mutation was first noted in a working (plaque purified) laboratory virus stock derived from the original ’76 Mayinga strain and does not affect virus growth in infected tissue culture cells.

To insert the eGFP open reading frame (ORF), a *Bsi*W1 restriction site was introduced into the NP 3′ UTR by inserting the nucleotides 5′-AGC-3′ between nucleotides 2851 and 2852. This insertion was performed using PCR to generate two overlapping fragments of ebolavirus sequence with a common *Bsi*W1 site which were subsequently ligated into the NP-GP subclone using the *Nde*1 and *Xho*1 sites in NP and VP35, respectively. The primers Ebo2407(+) 5-CTCTTTTGAGGAGATGTATCG-3′ and NP3′NCR*Bsi*W1(-) 5′-GTATGACGTACGGCTAGTA-ATAATAAGG-3′ were used for the first fragment, while the second fragment was amplified by using the primers NP3′NCR*Bsi*W1(+) 5′-GACTCTCGTACGTTTT-CAAAGTTCAATTTGAG-3′ and Ebo4105(-) 5′-ATACC-CAACCTCGATCAATC-3′. The eGFP ORF was then inserted into the newly introduced *Bsi*W1 restriction site present in the NP 3′ UTR along with an approximately 90 nucleotide duplicate set of mRNA transcription start (3′-CGCUACUUCUAAUU-5′) and poly A (3′-UAAUU-CUUUUUU-5′) signals normally located between the VP40 and GP ORFs. The 90-nucleotide fragment was generated by annealing the oligo 5′GTACGTGCCCTTC-TAATTAAGAAAAAATCGGCGATGAAGATTAAGCCG-ACAGTGAGCGTAATCTTCATCTCTCTTAGATTA-TTTGTTTTC 3′ and the oligo 5′CATGGAAAACAAAT AATCTAAGAGAGATGAAGATTACGCTCACT-GTCGGCTTAATCTTCATCGCCGATTTTTTCTTAATTA-GAAGGGCAC 3′. When annealed, the oligos had overhangs compatible with *Bsi*W1 and *Nco*1. The eGFP DNA fragment was amplified by PCR using the oligos *Nco*1eGFP(+) 5′ TACCGGTCGCCACCATGGTGAG-CAAGG 3′and *Bsi*W1eGFP(-) 5′ TAATACCGTACGTT-ACTTGTACAGCTCGTC 3′ to place a *Nco*1 and *Bsi*W1 site at the N and C-terminus of eGFP, respectively. The eGFP fragment along with the 90-nucleotide cassette was ligated into the *Bsi*W1 site present in the NP-GP subclone.

Virus was rescued by transfecting a 50/50 mixture of 293T and Vero E6 cells as previously described (Neumann et al., 2002). CPE generally appeared within 5–9 days after the passage of the transfected cell supernatants. Expression of eGFP was monitored by fluorescence microscopy.

Viruses and cell culture

The wild type '76 Mayinga strain of Zaire ebolavirus used in the single-cycle growth analysis was passaged three times in Vero E6 cells. The mouse adapted Zaire ebolavirus has been described elsewhere (Bray et al., 1999). EboV rescued from full-length clones were passaged 1–2 times in Vero E6 cells.

Single step growth analysis

Vero E6 cells were seeded in 24-well dishes using one 24-well dish for each virus tested. Cells were infected at a MOI of 2. After an initial 2 h adsorption at 37 °C in 1 ml of media (DMEM/2% FCS), each well was washed two times with PBS followed by the addition of 1 ml of media. The plates were then incubated at 37 °C. For each of the indicated time points, duplicate wells were harvested by adding 10 μl of 1 M Tris pH 8 and removing approximately 500 μl (in duplicate 250 μl aliquots) of supernatant and freezing at −80 °C. After all the time points were collected, the virus titers were determined by immunoplaque assay as previously described (Towner et al., 2004).

Infection of human PBMCs

Apharesis from healthy donors was layered on Ficoll gradient medium (ICN-biomedical Inc., Aurora, OH) in 1:1 ratio to isolate peripheral blood mononuclear cells (PBMCs). Purified PBMCs were plated in a 24-well plate (3×10^6 cells/well) and left for plate adherence at 37 °C for 1 h in a CO_2 incubator. Adherent cells were infected with 1 MOI of EboZ-eGFP virus (or medium for control wells) for 45 min at 37 °C with rocking followed by removal of non-adsorbed virus and subsequent add-back of non-adherent cells. Cells were examined for eGFP expression at 24, 48, 72, and 96 h post-infection. EGFP-expressing cells were analyzed for cell surface markers by labeling with either phycoerythrin (PE) or allophycocyanin (APC) conjugated antibodies specific for CD14, HLA-DR, CD19, and CD3. Cells were acquired on FACS Caliber and analyzed on Cell Quest software.

For electron microscopy, infected and uninfected PBMCs were pelleted, fixed, and embedded by standard methods and analyzed (Goldsmith et al., 2003).

Drug screening assay

The GFP-based virus drug discovery assay is a modification of previously described 96-well plate assays. In vitro cell cultures of Vero E6 cells in 96-well plates were infected with EboZ-GFP or control virus (Zaire ebolavirus-mouse adapted) at an MOI of 0.1. The test antiviral compound was then added to infected cultures in a range of dilutions designed to flank the predicted IC50. The IC50 was determined using a 4 parameter logit algorithm. Every 24 h for 7 days post-infection, plates were stained with neutral red or read in the fluorescent plate reader set at 450 nm (emission) and 515 nm (excitation). The neutral red was added to cells at a concentration of 1.11 mg/ml dissolved in PBS. After a 2-h incubation, cells were washed two times with ice-cold PBS. The retained stain was solubilized by adding 100 μl of a 1:1 mixture of 0.001 M ammonium phosphate and ethanol. An optical density was obtained at a wavelength 450 nm using a plate reader.

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