# DEVELOPMENT AND APPLICATION OF REVERSE GENETIC SYSTEMS FOR *RESTON EBOLAVIRUS*

BY

ALLISON GROSETH

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

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# DEDICATION

This thesis is dedicated to my family, who taught me the value of education, and who have been a boundless source of love and encouragement for me through this and all my other endeavours.

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There are a so many people and agencies without whom the completion of this stage of my training would not have been nearly so rewarding an experience.

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To **Ramon Flick**: what can I say? You were the first master of reverse genetics I ever met and I still believe that nobody makes a minigenome like you do. So, to say thanks for getting me hooked next time I'll buy the ice cream!

To **Hideki Ebihara**, all I can say is WOW! I don't know what I would have done without you...

私は、あなたの知識、価値観、科学に対する姿勢に大いに助けられたと

同時に、鼻っぱしをへし折られ、平伏させられました。それも良い経験

でした。だから、あなたに教えられた学び続ける事の大切さと、

このようなすばらしい先輩に師事できたことの幸運を心に留めてがんばって

いきたいと思います。

To **Victoria Wahl-Jensen**, I bet you thought I forgot you. No Way! You have been the best friend I could ever have asked for through what have been some of the best and some of the worst times in my life and I could never have made it without you. Thanks good buddy!

And last, but never least, I am incredibly grateful to **Thomas Hoenen**. You have been a pleasure to work with and an even greater pleasure to share my life with. I feel that I have learned so much from you as both a colleague and a companion and I look forward to continuing both these experiences during every stage of our lives.

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# ABSTRACT

## DEVELOPMENT AND APPLICATION OF REVERSE GENETIC SYSTEMS FOR *RESTON EBOLAVIRUS*

by

Allison Groseth

University of Manitoba, March 2006.

The genus *Ebolavirus*, in the family *Filoviridae*, is divided into four species: *Zaire*, *Sudan*, *Ivory Coast* and *Reston*. With the exception of *Reston ebolavirus* (REBOV), all filoviruses cause severe haemorrhagic fever in both human and non-human primates. A molecular basis for this difference in pathogenicity has not yet been established.

Preliminary work for this study involved sequence determination for REBOV. As expected, based on the dramatic phenotypic differences, the full-length sequence of REBOV differed substantially at both the nucleotide and amino acid levels from more pathogenic filoviruses; however, it is difficult to speculate on the role of such differences, particularly in light of the limited functional data available for filovirus proteins. Based on these data we were able to develop a minigenome system for REBOV, which models many aspects of the viral lifecycle (i.e. encapsidation, transcription, replication and packaging). Using this system it was observed that the ability of the RNP proteins to mediate transcription from a given template correlated with the *in vivo* pathogenicity of the virus from which they were derived. Data also suggest that the transcriptional requirement for VP30 by Ebola viruses may be dependent on characteristics of the RNP

complex, and not solely on the presence of genomic hairpins, which have been previously reported to play a role in this process.

We have also applied our minigenome system as a screening tool for siRNAs that inhibit transcription/replication of either REBOV, or the highly pathogenic *Zaire ebolavirus* (ZEBOV). We found that the ZEBOV siRNAs were also capable of inhibiting virus growth in cell culture as well as in a mouse model of infection. Here, treatment with siRNA decreased weight loss, a sensitive indicator of illness, suggesting that further testing in a lethal model is warranted and that siRNAs have potential for development as a therapeutic measure.

Finally, based on information obtained during this study, an infectious clone system for REBOV is now being constructed. Once this system becomes available, it will be possible to generate REBOV entirely from cloned cDNA. This will make it possible to study recombinant viruses and thereby allowing us to determine the role of different virus proteins and/or genomic elements in pathogenesis, particularly when complementary recombinants are evaluated in the pathogenic ZEBOV infectious clone system.

# 1.0 Introduction

## 1.1 *Filoviridae*

### 1.1.1 Taxonomy and Nomenclature

Together with the members of the families *Paramyxoviridae*, *Bornaviridae* and *Rhabdoviridae*, the family *Filoviridae* makes up the order *Mononegavirales*, which contains all non-segmented, negative-sense, single-stranded (NNS) RNA viruses. Since it was first distinguished from the family *Rhabdoviridae* in 1982 (Kiley et al., 1982) and re-classified as a separate family, there have been a number of tentative taxonomic assignments given to the filoviruses. The most recent classification, as described in the 8th Report of the International Committee on Taxonomy of Viruses (Feldmann et al., 2004) divides *Filoviridae* into two genera, *Marburgvirus* and *Ebolavirus*. While the genus *Marburgvirus* consists of a single species, *Lake Victoria marburgvirus* (MARV), the genus *Ebolavirus* (EBOV) is subdivided into four species, *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Ivory Coast ebolavirus* (ICEBOV), and *Reston ebolavirus* (REBOV) (Table 1).

For both *Marburgvirus* as well as the four *Ebolavirus* species a number of strains have been identified with those recognized by the International Committee on Taxonomy of Viruses (ICTV) being listed in Table 1. Apart from an obvious phylogenetic division between the two genera based on nucleotide sequence (Figure 1), they are further distinguished by their general lack of antigenic cross-reactivity and differences in their genome organization, particularly with respect to the number and position of gene overlaps and the presence or absence of an RNA editing site in the glycoprotein (GP) gene (Figure 2).

**Table 1. Taxonomic classification of the family *Filoviridae*.**

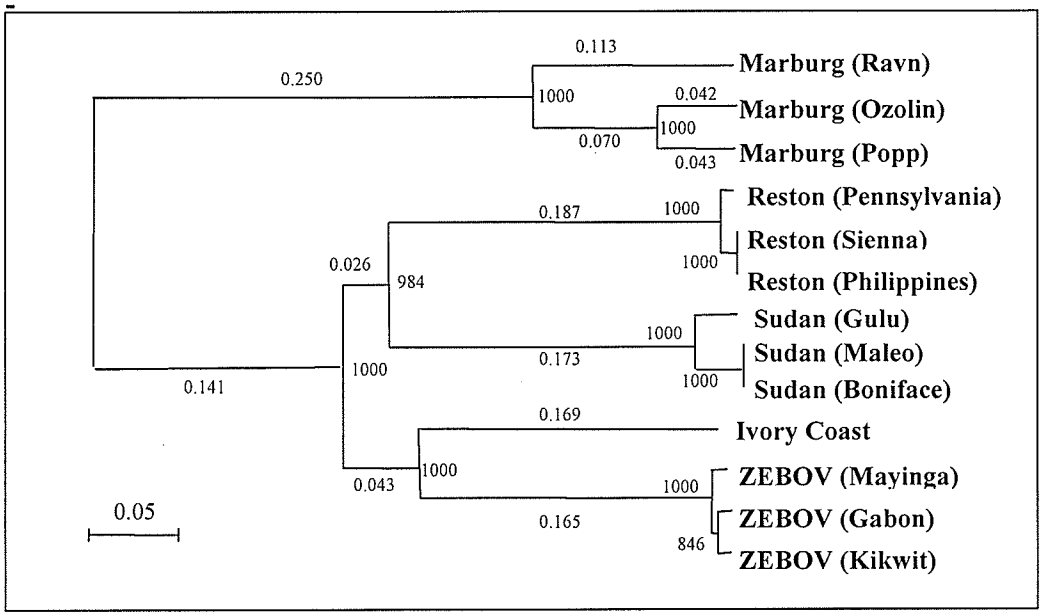
| Genus | Species | Strain* (Location, Year) | Genbank Accession Number ¥ |
|---|---|---|---|
| *Marburgvirus* | *Lake Victoria marburgvirus* (MARV) | Ratayczak (Germany, 1967) | ---- |
| | | Popp (Germany, 1967) | Z29337 |
| | | Voege (Yugoslavia, 1967) | ---- |
| | | Ozolin (Zimbabwe, 1975) | AY358025 |
| | | **Musoke (Kenya, 1980)** | **Z12132** |
| | | Ravn (Kenya, 1987) | ---- |
| | | Angola (Angola, 2005) | ---- |
| *Ebolavirus* | *Zaire ebolavirus* (ZEBOV) | **Mayinga (Zaire, 1976)** | **J04337** |
| | | Zaire (DRC, 1976) | ---- |
| | | Eckron (DRC, 1976) | ---- |
| | | Tandala (DRC, 1977) | ---- |
| | | Gabon (Zaire, 1994) | ---- |
| | | Kikwit (DRC, 1995) | AY354458 |
| | *Sudan ebolavirus* (SEBOV) | Boniface (Sudan, 1976) | ---- |
| | | Maleo (Sudan, 1979) | ---- |
| | | Gulu (Uganda, 2000) | AY729654 |
| | *Ivory Coast ebolavirus* (ICEBOV) | **Cote d'Ivoire (Ivory Coast, 1994)** | ---- |
| | *Reston ebolavirus* (REBOV) | **Reston (USA, 1989)** | ---- |
| | | **Pennsylvania (USA, 1989)** | **AF522874, AY769362** |
| | | Siena (Italy, 1992) | ---- |
| | | Texas (USA, 1996) | ---- |
| | | Philippines (Philippines, 1996) | AB050936 |

DRC = Democratic Republic of the Congo
¥ Genbank accession numbers are shown for complete genome data only
* Prototype strains for each species shown in bold-faced type.

### 1.1.2 *Reston ebolavirus*

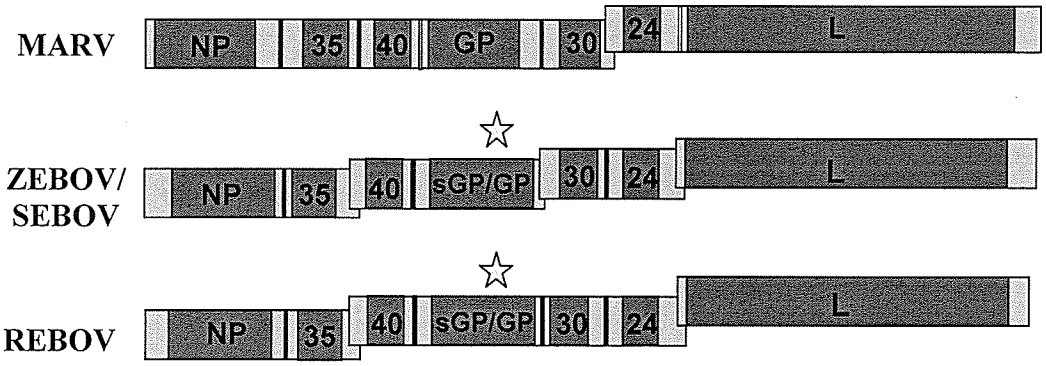
The Reston species of *Ebolavirus* is unique in that it originated in Asia, while the other filoviruses have their origins in Africa (Feldmann & Klenk, 1996, Miranda et al., 1999). It emerged in 1989/90 as the causative agent of an epizootic among a group of cynomolgus macaques (*Macaca fascicularis*) imported from the Philippines into the United States (Jahrling et al., 1990). Subsequently, at least two more introductions of REBOV have occurred in the United States and Italy (Rollin et al., 1999, WHO, 1992).

**Figure 1. Phylogenetic classification of filoviruses.** Based on phylogenetic analysis of the family *Filoviridae* a clear division can be seen between the two genera, *Marburgvirus* and *Ebolavirus*. As well, the divisions between the four Ebola virus species are clearly visible. The bootstrap proportions are shown at nodes while the distances along each branch indicate mutations per 100 nucleotides.




Epidemiological investigations in the Philippines have documented active virus transmission in a single export facility that was the source of all the infected monkey shipments (Hayes et al., 1992, Miranda et al., 1999). Despite its significant pathogenicity for nonhuman primates, REBOV has never been associated with any notable disease in man. However, serological investigations have documented at least eight seroconversions among exposed animal handlers (CDC, 1990a, CDC, 1990b, Miranda et al., 1999, Miranda et al., 1991), which suggests that REBOV infections lead to either asymptomatic or subclinical courses of disease in humans. Furthermore, there is some data suggesting that REBOV also displays decreased virulence for nonhuman primates (Fisher-Hoch et al., 1992). Therefore, REBOV is an interesting species with which to study the pathogenicity of filoviruses, particularly in comparison to ZEBOV, the most virulent species.

**Figure 2. Genome organization of filoviruses.** The gene orders of fully sequenced filovirus genomes are presented. The intergenic regions are shown in black, the non-coding regions in grey and the open reading frames in red. Steps indicate the positions of the gene overlaps and stars indicate the position of the RNA editing site in the EBOV GP gene.




[Altered from (Groseth et al., 2002)]

## 1.1.3 Biohazard Classification

Despite the lack of evidence for any disease associated with REBOV infection, all members of the *Filoviridae* are classified as "Risk Group 4" agents (LCDC, 2004). This classification indicates that these pathogens pose both a high individual and a high community risk as a result of their ability to cause very serious, highly transmittable, untreatable human and/or animal disease. As such, all work with infectious materials containing or suspected to contain filoviruses must be performed in a biosafety level 4 (BSL 4) facility.

## 1.1.4 Genome Organization

The single negative-sense linear RNA genome of filoviruses does not contain a poly(A) tail and is non-infectious (Regnery et al., 1980). Filovirus genomes are approximately 19 kb in length (19.1 kb for MARV; EBOV 18.9 kb), with the genes

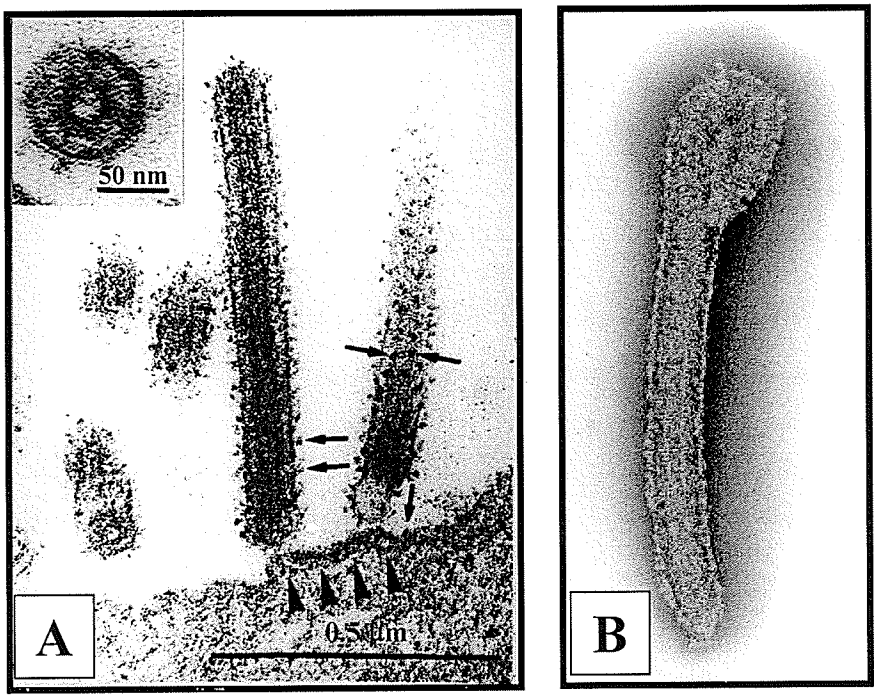
organized in the following linear order: *3' leader – NP – VP35 - VP40 – GP - VP30 - VP24 – L - 5' trailer* (Bukreyev et al., 1995, Feldmann et al., 1992, Ikegami et al., 2001, Sanchez et al., 1993) (Figure 2). Genes are delineated by transcriptional signals at their 3' and 5' ends that have been identified by their conservation and by sequence analysis of mRNA species. The sequences *3'-CUNCNUNUAAUU-5'* and *3'-UAAUUCUUUUU-5'* represent the consensus motifs for transcriptional start and stop signals respectively (Muhlberger et al., 1996, Sanchez et al., 1993, Sanchez et al., 1989, Sanchez et al., 1992). Intergenic regions, which vary in length as well as nucleotide composition, usually separate filovirus genes from each other, but some gene overlaps exist at characteristic positions (Figure 2). The length of the overlaps is limited to five highly conserved nucleotides within the transcriptional signals, 3'-*UAAUU*-5', located at the internal ends of the conserved sequences (i.e. CUNCNUNUAAUUCAAAAA). Most genes tend to possess long non-coding sequences at their 3' and/or 5' ends, which contribute to the increased length of the genome. Extragenic sequences are found at the 3'- leader and 5'-trailer ends of the genome and are complementary to each other at the extreme ends, a feature that is shared by many NNS RNA viruses (Feldmann & Kiley, 1999, Sanchez et al., 2001).

### 1.1.5 Virion Morphology

Filoviral particles are filamentous in shape, but can also appear as branched, circular, U- or 6-shaped forms (Figure 3). They display a uniform diameter of approximately 80 nm, but vary greatly in length. Negatively contrasted particles, regardless of strain, contain an electron-dense central axis (19-25 nm in diameter) surrounded by an outer helical layer (45-

50 nm in diameter) with cross-striations at 5 nm intervals. This central core, which is formed by the RNP complex, is further surrounded by a host cell plasma membrane-derived lipid envelope in which glycoprotein spikes are embedded. These spikes, which are visible as globular structures on the surface of virions (Figure 3), are ~7 nm in diameter and spaced at 5-10 nm intervals (Geisbert & Jahrling, 1995, Murphy et al., 1971).

**Figure 3. Filovirus particle structure. (A) Marburg virus structure.** The particles shown here demonstrate the characteristic filamentous shape. The electron-dense central axis, formed by the ribonucleoprotein complex is visible in the inset and sites of budding are indicated by the arrow heads. **(B) Ebola virus structure.** The Ebola virus particle shows a common 6-shaped form. The lipid envelope is clearly visible in both images with the glycoprotein (GP) seen as projections on the surface of the particles (indicated by arrows in panel A).




**[Panel A altered from (Schnittler et al., 1993) Panel B kindly provided by L. Kolesnikova]**

## 1.1.6 Viral Proteins

Virus particles are made up of seven structural proteins with presumed identical functions for the different viruses (Table 2). Four proteins, together with the viral RNA,

**Table 2. Summary of filovirus proteins and their functions.**

| Protein | Presence in EBOV | Presence in MARV | Virion Localization | Function(s) |
|---|---|---|---|---|
| NP | + | + | RNP complex | RNA encapsidation, Regulation of transcription/replication |
| VP35 | + | + | RNP complex | Polymerase cofactor, IFN-antagonist |
| VP40 | + | + | Matrix space | Matrix protein |
| $GP_{1,2}$ | + | + | Envelope | Receptor binding, Fusion |
| $GP_{1,2}\Delta TM$ | + | ? | Non-structural (Shed by TACE cleavage) | Unknown |
| $GP_1$ | + | + | Non-structural (Secreted) | Unknown |
| sGP | + | - | Non-structural (Secreted) | Unknown |
| Δ-peptide | + | - | Non-structural (Secreted) | Unknown |
| VP30 | + | + | RNP complex | Transcription activator, Early transcriptional anti-terminator |
| VP24 | + | + | Matrix space | Minor matrix protein, Nucleocapsid formation, IFN antagonist |
| L | + | + | RNP complex | RNA-dependent RNA polymerase (RdRp) |

make up the RNP complex [nucleoprotein (NP), virion protein (VP) 35, VP30 and RNA-dependent RNA polymerase (L)] (Elliott et al., 1985). These four proteins represent the minimal necessary factors for the transcription and replication of filoviruses, although VP30 has been shown to be dispensable for replication alone (Muhlberger et al., 1999). The remaining three proteins; glycoprotein ($GP_{1,2}$), VP40 and VP24, are membrane-associated and function as the surface glycoprotein, major matrix protein and minor matrix protein, respectively (Elliott et al., 1985, Han et al., 2003, Jasenosky et al., 2001). In addition, Ebola viruses express several non-structural glycoprotein products, although no definitive functions have yet been assigned to them (Dolnik et al., 2004, Volchkov et al., 1998b, Volchkova et al., 1998, Volchkova et al., 1999, Wahl-Jensen et al., 2005a).

### 1.1.6.1 Nucleoprotein

The most 3' gene in the filovirus genomic RNA encodes a nucleoprotein of approximately 105 kD, almost twice the size of those encoded by other members of *Mononegavirales*. While singular expression of the nucleocapsid protein results in the formation of large inclusion bodies into which other RNP complex proteins can be recruited (Becker et al., 1998), it has been shown recently that NP, VP35 and VP24 are sufficient for the formation of nucleocapsids, a process which was reported to be dependant on post-translational O-glycosylation and sialation of the nucleoprotein (Huang et al., 2002). However, no mechanism by which the large glycosylations reported could occur, given the cytoplasmic localization of the nucleoprotein, has been identified. As a result, the possibility of NP glycosylation is still much debated within the field. It has, however, been reliably reported that phosphorylation of the nucleoprotein occurs at serine and threonine, but not tyrosine residues, both when isolated from virions and when expressed in eukaryotic systems (Lotfering et al., 1999).

Despite the presence of many potential phosphorylation sites in the N-terminal half of NP, only the C-terminal portion of the protein is actually phosphorylated. Many of these phosphorylated residues fall into consensus recognition sequences for either protein kinase CKII or proline-directed protein kinases. Unfortunately, to date there is no function associated with these phosphorylation events. However, it is known that loss of phosphorylation at the 3 proline-adjacent serine residues does not affect transcription or replication, indicating that at least serine phosphorylation of NP is not likely to be a regulatory factor for viral RNA synthesis (Lotfering et al., 1999).

### 1.1.6.2 Virion Protein 35

The second gene in the EBOV genome encodes a phosphoprotein (P) homologue known for filoviruses as VP35. In contrast to many other P proteins, VP35 appears to be only weakly phosphorylated (Becker & Muhlberger, 1999). However, like other P proteins, VP35 is a structural component of the viral RNP complex and is an essential factor for viral transcription and replication where it functions as an RNA-dependent RNA polymerase (RdRp) cofactor (Muhlberger et al., 1999). Recently, it has been shown that interaction through a predicted coil-coil domain between amino acids 70 and 120 is both necessary and sufficient to mediate this oligomerization and that this process is essential for the function of VP35 as a polymerase cofactor (Moller et al., 2005). Based on colocalization data it also appears that oligomerization of VP35 is necessary for interaction with L, but not for interaction with NP (Moller et al., 2005). Thus the relationship between oligomerization and its cofactor function likely derives from the function VP35 plays as a bridging molecule facilitating interaction between NP and L and ultimately recruitment of the polymerase to the RNA template.

Similar to other viruses, EBOV is capable of evading the type-I interferon (IFN) antiviral response by encoding IFN antagonists. In ZEBOV this process of IFN antagonism is mediated by VP35 (Basler et al., 2000). It has been shown that EBOV VP35 inhibits initial type-I IFN production by interfering with phosphorylation of IRF-3, which is necessary for dimerization, nuclear translocation and subsequently for the transcription of IFN genes (Basler et al., 2003, Basler et al., 2000). Furthermore, it was shown that the C-terminal region of VP35 has a double-stranded RNA (dsRNA)-binding motif similar to the influenza A virus NS1 protein, another IFN antagonist, (Hartman et

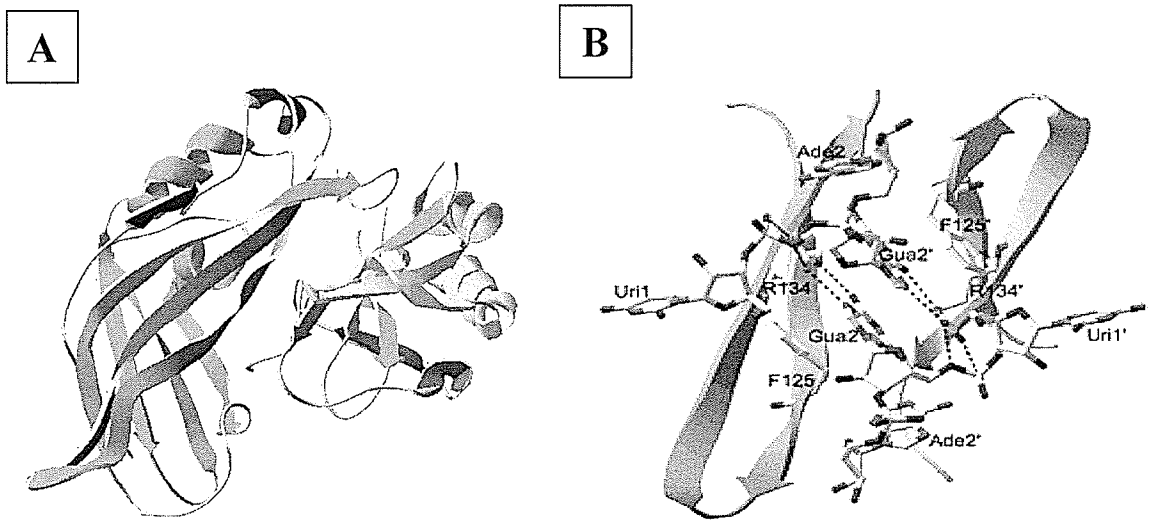
al., 2004), and that disruption of this motif leads to a decrease of the anti-IFN activity of VP35 (Hartman et al., 2004). Similar to the polymerase cofactor function of VP35, its IFN-antagonist function has also been shown to be dependant on oligomerization through the coiled-coil motifs in the amino terminus of VP35 and that these interactions serve to enhance the IFN-antagonist function exerted by the carboxyl-terminal half (Reid et al., 2005).

### 1.1.6.3 Virion Protein 40

VP40, encoded by the third gene, has been identified and characterized as the major matrix protein. It is believed to play a central role in viral assembly by targeting to and interacting with the plasma membrane and ultimately forming a tightly packed layer on the inner side of the viral envelope. Perhaps as a result of this function, it is the most abundant of the proteins in both EBOV and MARV particles.

Much of our knowledge about how VP40 carries out these functions derives from X-ray crystallographic data generated by Dessen et al (Dessen et al., 2000a, Dessen et al., 2000b). This work demonstrated for the first time that, not only is VP40 distinct from all other viral matrix proteins based on its sequence, but it is topologically distinct. It consists of two domains with similar β-sandwich structures, connected by a flexible linker, suggesting that these domains may have been generated by gene duplication (Dessen et al., 2000a, Dessen et al., 2000b) (Figure 4A). The connection of these domains by a relatively disordered linker region further suggests the possibility that conformational change(s) and/or domain movement may be important for VP40 function

**Figure 4. Structural and functional domains of VP40. (A) Crystal structure of monomericVP40.** VP40 shows a distinctive two-domain structure joined by a flexible linker. Each domain has a β-sandwich and these domains move around their linker to separate these domains and thereby mediate oligomerization into either hexamer or octamers. **(B) Interaction of octameric VP40 with RNA.** In addition to domain movement, octamerization of VP40 required the presence of RNA containing the triribonucleotide 5'-UGA-3' which interacts with F125 and R134 to stabilize the intradimeric interactions.




**[Panel B altered from (Hoenen et al., 2006)]**

(Dessen et al., 2000a, Dessen et al., 2000b). Further substantiating this idea is the observation that C-terminal truncations, destabilizing urea treatment or membrane interaction are all sufficient to induce the formation of hexamers in solution (Ruigrok et al., 2000, Scianimanico et al., 2000). Based on the crystal structure it was further observed that the C-terminal domain contains large solvent-exposed hydrophobic patches, which correlates with previous experimental data indicating that this domain is absolutely required for membrane binding. As a result of these observations it possible that the initiation of budding occurs through the following process: (i) VP40 binds to lipid bilayers, likely in multivesicular bodies (Kolesnikova et al., 2004), through its C-terminal domain using a combination of hydrophobic and electrostatic interactions; (ii) this results in a destabilization of VP40 which induces a conformational change involving movement

of the C-terminal domain away from the N-terminal domain; (iii) the N-terminal domain is then free to hexamerize into ring-like structures; (iv) these hexameric building blocks may then undergo further oligomerization to form a lattice underlying the plasma membrane through bridging interactions with the VP40 C-terminal domain and/or other viral proteins.

Consistent with its role as the major matrix protein, it was finally demonstrated that VP40 provides the force for virus particle budding. This was shown by its ability, when expressed alone from transfected plasmid, to produce filamentous virus-like particles (VLPs) similar to authentic virus particles when examined by electron microscopy (Noda et al., 2002, Timmins et al., 2001). The observation that these particles closely resemble those produced by virus infection further shows that VP40 dictates the unique morphology associated with filoviruses. This ability of VP40 to mediate budding has recently been further linked to the presence of proline-rich motifs (i.e. PT/SAPP and PPXY) in the N-terminus of the protein (Harty et al., 2000, Jasenosky et al., 2001, Licata et al., 2003, Martin-Serrano et al., 2001, Timmins et al., 2003, Yasuda et al., 2003). Harty and colleagues were the first to establish that VP40 was capable of binding to WW-domain proteins, particularly Nedd4, through the PPXY motif (Harty et al., 2000), however, it was not until later that Jasenosky showed that mutation of the PPXY motif blocked budding of VLPs (Jasenosky et al., 2001), suggesting that this process most likely relies on interaction with cellular WW domain-containing proteins. Since this time Tsg101 and Vps4, both components of the vacuolar sorting pathway, have been identified as additional functional binding partners for VP40 (Licata et al., 2003, Martin-Serrano et al., 2001, Yasuda et al., 2003) and the E3 ligase function of Nedd4 has

been identified as being crucial for its effect on budding (Yasuda et al., 2003). For Nedd4, is was shown that binding to the PPXY motif requires an oligomeric conformation of VP40, while Tsg101 binding to the PT/SAP motif occurs regardless of the oligomeric state of the matrix protein (Timmins et al., 2003), indicating that these binding events may play complementary roles in budding at different stages of assembly. However, these domains were recently shown, using the infectious clone system, to be non-essential for virus growth in cell culture, where only slight decreases in virus growth were seen in the absence of one or both of the proline-rich domains (Neumann et al., 2005). This clearly indicates that there are alternative pathways for virus budding, which are proline-rich domain independent.

Recently, another oligomeric form of VP40 not associated with the budding process has been identified by X-ray crystallographic studies. This octameric form of VP40 is composed of four anti-parallel homodimers, which bind a 5'-UGA-3' RNA triribonucleotide at their inner pore surface through interaction with the residues F125 and R134 (Figure 4B) (Gomis-Ruth et al., 2003). This selective RNA interaction stabilizes the octameric ring structure and confers SDS resistance to the structure. The presence of this octameric form of VP40 in Ebola virus-infected cells (Gomis-Ruth et al., 2003) as well as in virus-like particles (Hoenen et al., 2006), suggests an additional function in the virus life cycle. This idea has been recently confirmed by the inability of researchers to rescue ZEBOV that contains mutations that abolish RNA binding, and thus oligomerization, using the reverse genetics system (Hoenen et al., 2006). While no clear function has yet been assigned to these octamers, it has been determined that they have no apparent effect on VLP budding (Hoenen et al., 2006) and recent work indicates that

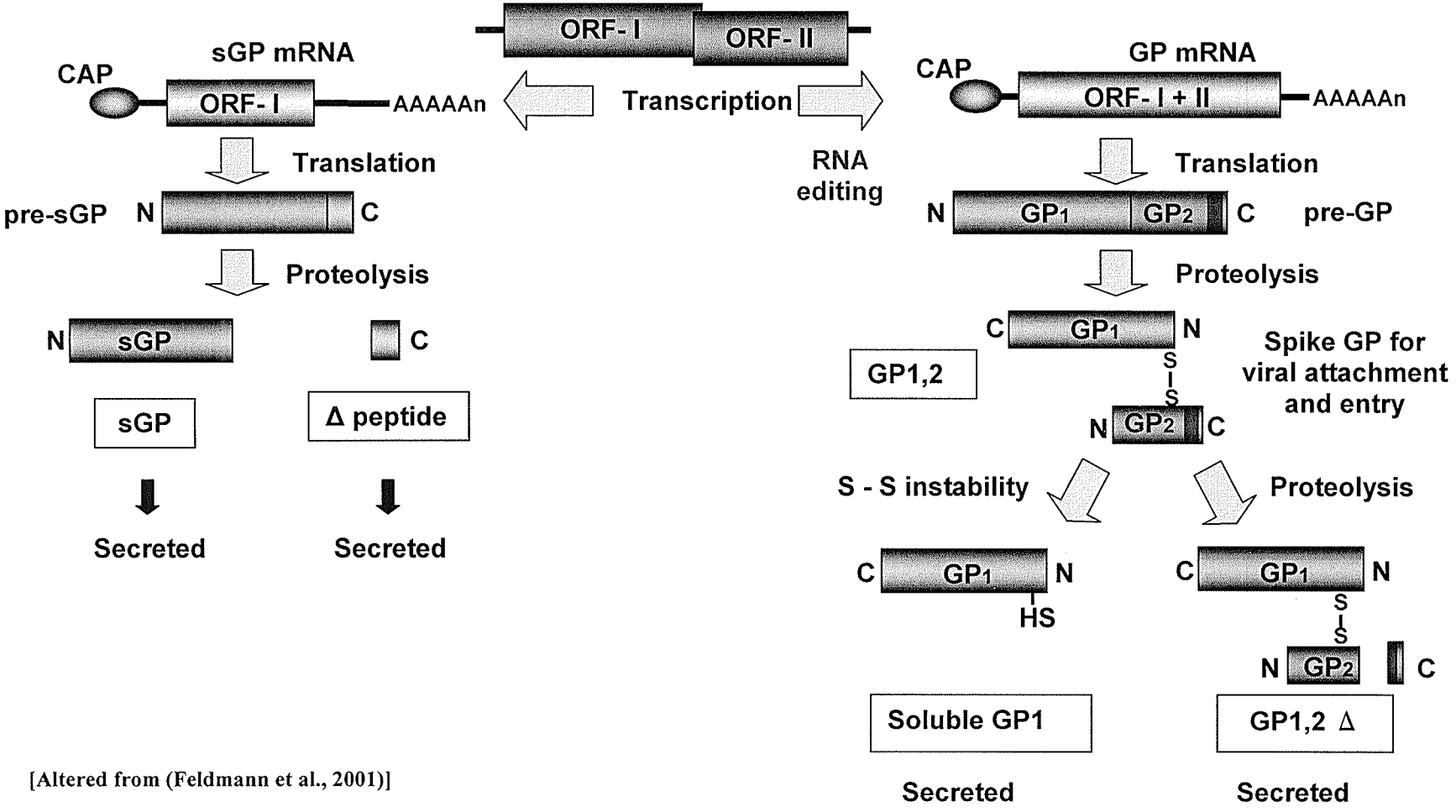
they may impact on the generation and/or incorporation of functional RNPs into virus particles (T. Hoenen, personal communication).

### 1.1.6.4 Glycoprotein

The viral glycoprotein, GP, has to date been the most intensively studied of the filovirus proteins. It mediates viral entry, including attachment and membrane fusion, and plays an important role in pathogenesis and host immune response (Feldmann et al., 2001, Ito et al., 1999, Ito et al., 2001, Takada et al., 2000, Takada et al., 2001, Watanabe et al., 2000).

In contrast to the closely related MARV, EBOV demonstrates a unique glycoprotein expression strategy in which the glycoprotein products, encoded by the fourth gene of the viral genome, are produced from two overlapping ORFs (Figure 5). The predominant products of this gene for all EBOV species are the soluble secreted glycoproteins sGP and Δ-peptide, a small carboxyl-terminal peptide generated through cleavage by furin or a furin-like endoprotease from a precursor (pre-sGP) (Sanchez et al., 1998, Volchkov et al., 2000b, Volchkova et al., 1999). Both of these products are efficiently secreted from infected cells but their functions remain unknown, this is despite some conflicting reports indicating that sGP may or may not play a role in immune evasion by binding to and inactivating neutrophils, or acting as a decoy for neutralizing antibodies directed against $GP_{1,2}$ (Ito et al., 2001, Sui & Marasco, 2002, Yang et al., 1998). In contrast to sGP, the transmembrane $GP_{1,2}$, which is found as a homotrimer on the surface of mature EBOV particles (Sanchez et al., 1998), is only produced through transcriptional RNA editing (Sanchez et al., 1996, Volchkov et al., 1995), while that

**Figure 5. Glycoprotein expression strategies for Ebola virus.** The products sGP and Δ-peptide are produced by direct transcription of the GP ORF-I, the translation of which produces a pre-sGP precursor. This precursor is converted into the mature proteins through proteolytic processing by furin. In contrast, the remaining products are generated through transcriptional editing involving insertion of an additional A residue near the end of ORF-I, thus facilitating a shift into ORF-II, the translation of which generates the pre-GP precursor. Proteolytic processing of pre-GP by furin yields the mature structural glycoprotein GP1,2 which can be converted to GP1,2 Δ by TACE cleavage at the cell surface or lead to the release of GP1 through reduction of the disulphide bond joining GP1 to the transmembrane fragment.




[Altered from (Feldmann et al., 2001)]

produced by MARV results from direct transcription and translation of the single open reading frame (Will et al., 1993) (Figure 5). RNA editing is performed by the viral RdRp (L) and occurs at a seven-adenosine editing site as a result of a single adenosine insertion and controls the ratio of sGP to GP expression in the infected cell (Sanchez et al., 1996, Volchkov et al., 1995). Translation of the edited EBOV mRNA transcript results in a precursor, pre-GP, which is proteolytically cleaved into a larger N-terminal ($GP_1$) ectodomain and smaller C-terminal ($GP_2$) subunit containing the proposed fusion peptide, the membrane-spanning transmembrane (TM) domain, and a short cytoplasmic tail. This cleavage event occurs at a multiple basic site and seems to be mediated by furin (Sanchez et al., 1998, Volchkov et al., 1998a, Volchkov et al., 2000b). As is seen in Table 3, the cleavage site in the REBOV GP, unlike that of other filoviruses, deviates from the consensus motif for furin cleavage (R-X-R/K-R; X = any amino acid).

**Table 3. Proteolytic cleavage sites of filovirus glycoproteins and their correlation to virus pathogenicity.**

| Virus Species | $GP_{1,2}$ Furin Cleavage Site | Pathogenicity | |
|---|---|---|---|
| | -4 -3 -2 -1 | Human | Monkey |
| ZEBOV | - R - T/A - R - R - | +++ | +++ |
| SEBOV | - R - S - R - R - | +++ | ++ |
| ICEBOV | - R - K - R - R - | + / ++ * | + (++) |
| REBOV | - K - Q - K - R - | - ¥ | + / ++ |
| MARV | - R - L - R - R- | ++ (+++)§ | ++(+++)§ |

* Based on only two cases of human ICEBOV infection that have been reported to date.
¥ Data indicate an absence of disease in infected humans; however, the sample size is currently small.
§ Recent outbreaks of MARV in Africa have resulted in case fatality rates much higher than those observed in the initial 1967 outbreak. These values, which are in the range of 75-90%, suggest that the pathogenic potential of MARV is equal to that of ZEBOV.

**[Altered from (Feldmann et al., 2001)]**

Since in other enveloped viruses (e.g.. avian influenza virus), proteolytic cleavage of the envelope glycoprotein has been shown to be one of the key factors for virus infectivity and pathogenicity (Horimoto & Kawaoka, 1994), it has been widely postulated that this may explain the reduced growth of REBOV in tissue culture, as well as its apparent reduction in virulence in humans and some non-human primates species (Feldmann et al., 2001). However, experiments using a furin cleavage deficient recombinant ZEBOV showed less than a 1 log reduction in virus titre from tissue culture, suggesting that cleavage may not be important for *in vitro* infectivity (Neumann et al., 2002a). While it remains to be seen whether furin cleavage will be a critical factor for *in vivo* virus growth, recent data indicate that furin cleavage may also be dispensable for pathogenicity in non-human primates (T. Geisbert, personal communication).

Two additional glycoprotein products can also be derived from the mature $GP_{1,2}$. In the mature spike protein the $GP_1$ and $GP_2$ subunits are disulfide linked (Sanchez et al., 1998, Volchkov et al., 1998a), however, during processing, these bonds in $GP_{1,2}$ can become unstable, releasing the non-membrane-bound fragment $GP_1$ which is then secreted from infected cells (Volchkov et al., 1998b). Recently, it was also shown that another soluble product, $GP_{1,2\Delta TM}$, can be produced through metalloprotease cleavage of the membrane-bound mature $GP_{1,2}$ at the cell surface (Dolnik et al., 2004).

While a direct function for sGP in the virus life cycle and/or pathogenesis remains unclear, a recombinant ZEBOV, produced using the infectious clone system and containing an altered RNA editing site that resulted in the preferential expression of $GP_{1,2}$ has provided insight into the role of sGP in the unique filovirus glycoprotein expression strategy (Volchkov et al., 2001). Compared to wild-type virus, the mutant virus

expressed increased levels of $GP_{1,2}$ and was more cytolytic, but was compromised in its tissue culture growth abilities, suggesting that transcriptional regulation of sGP and GP expression has an important role in avoiding inefficient multiplication due to the cytotoxicity of the accumulating $GP_{1,2}$ (Volchkov et al., 2001). This *in vitro* cytotoxicity of ZEBOV $GP_{1,2}$ is associated with cell rounding and detachment of cells through down-regulation of several cell surface molecules, including beta-integrin, alpha-V-integrin, epidermal growth factor receptor, transferrin receptor and MHC-1 (Simmons et al., 2002, Takada et al., 2000). Furthermore, several groups have shown that the Ser/Thr-rich mucin-like domain is important for this GP-mediated cytotoxicity (Simmons et al., 2002, Yang et al., 2000). Interestingly, this mucin-like domain of ZEBOV GP also has an important role in enhancing virus entry by binding human macrophage C-type lectins specific for galactose/N-acetylgalactosamine (hMGL) (Takada et al., 2004). Since hMGL is expressed on immature dendritic cells (DCs), this region may be one of the determinants for EBOV tropism.

#### 1.1.6.5 Virion Protein 30

VP30, the product of the fifth gene in the linear genome, is a component of the RNP complex and has been shown to be a potent activator of transcription for Ebola virus (Muhlberger et al., 1999). However, it shows no effect on MARV transcription (Muhlberger et al., 1998, Muhlberger et al., 1999), although a clear basis for this difference has yet to be established. Unlike NP, VP35 and L, which have conserved homologues throughout *Mononegavirales*, VP30 appears to be an EBOV-specific transcription factor. Using the plasmid based minigenome system it was further shown

that VP30 of ZEBOV promotes transcription by functioning as an early anti-termination factor (Weik et al., 2002). It has been predicted that the presence of VP30 might resolve or cover predicted RNA secondary structures either by RNA binding or by directing an additional co-factor to the folding RNA. However, thus far, RNA binding activity for VP30 has not been described and, since the viral genome is bound by NP, secondary structure formation may not occur. Since the only naked RNA species present are the positive-sense mRNA transcripts, which have been experimentally shown to form hairpins in the sequence upstream of and including the NP transcriptional starts site, it could be possible for VP30 to have an effect at the level of these structures (Weik et al., 2002). Our knowledge of VP30 was extended when VP30 was found to contain two N-terminal serine clusters, in the region from amino acids 40-51, whose phosphorylation positively regulated the binding of VP30 to NP ($Ser_{40}$ and $Ser_{41}$), and in doing so negatively regulated the transcription activation function of VP30 (Modrof et al., 2002). It was also shown that VP30 is a target for the cellular protein phosphatases PP1 and PP2A and consistent with a role for these proteins in the phosphorylation status of VP30, treatment of ZEBOV infected cells with okadaic acid, which inhibits PPI and PP2A, inhibited ZEBOV growth (Modrof et al., 2002). More recently an unconventional Cys(3)-His zinc-binding motif at amino acids 68 to 95 has been identified (Modrof et al., 2003). This motif was shown to stoichiometrically bind zinc ions in a one-to-one relationship, a process that was shown to be dispensable for nucleocapsid formation but critical for viral transcription (Modrof et al., 2003). Finally, a homoligomerization region of VP30 has been recently identified at amino acids 94-112 and consists of four critical leucine residues (Hartlieb et al., 2003). Mutation of one of these leucine residues resulted

in oligomerization-deficient VP30 molecules that were no longer able to support EBOV-specific transcription and disruption of homoligomerization using a synthetic 25-mer peptide derived from the oligomerization region could inhibit viral replication (Hartlieb et al., 2003).

### 1.1.6.6 Virion Protein 24

Little is known about EBOV VP24, which is encoded by gene six of filoviruses. However, it is considered to function as a minor matrix protein, with studies indicating that VP24 may also be involved in nucleocapsid formation (Huang et al., 2002, Noda et al., 2005). Based on biochemical studies, it is now known that VP24 localizes to the plasma membrane and perinuclear regions and associates strongly with lipid membranes (Han et al., 2003). Additionally, it was shown that VP24 is capable of forming oligomers in a process that seems to be mediated through the N terminus of the protein (Han et al., 2003). In light of the role of VP24 as a minor matrix protein, it was then interesting that its expression had no significant effect either on the release of VP40 in the form of virus-like particles (VLPs) (Licata et al., 2004), or on the release of infectious VLPs (Watanabe et al., 2004). However, Noda et al. were able to confirm that VP24 is critical for the formation of tubular, left-handed helical nucleocapsids resembling those produced during infection (Noda et al., 2005).

More recently, a role for VP24 as an IFN antagonist has been demonstrated (Basler & Palese, 2004, Halfmann & Kawaoka, 2005), with data indicating that inhibition of p38-MAPK may be involved in its ability to block IFN signalling. It is then interesting that both mouse and guinea pig-virulent EBOV strains are known to have

acquired mutations in VP24 during the adaptation process (Bray et al., 1998, Volchkov et al., 2000a). In mice, where the type-I interferon response is particularly important for resistance against filovirus infections (Bray, 2001) two single amino acid mutations in NP and VP24 were shown to be both necessary and sufficient for mouse adaptation (H. Ebihara, personal communication). Similarly, mutations in VP24 appear to be important for guinea pig adaptation where they have been identified in a number of adapted variants (Volchkov et al., 2000a). In addition, during the adaptation process in guinea pigs a decreased inflammatory response of infected mononuclear phagocytic cells as well as increased infection of other target cells (hepatocyte, endothelial cell, and fibroblast) were observed (Chepurnov et al., 2003, Ryabchikova et al., 1996), further substantiating the idea that VP24 may have an important role in the inhibition of innate immunity and the early inflammatory responses in infected animals.

### 1.1.6.7 RNA-Dependant RNA Polymerase

The seventh gene of the viral genome encodes the largest of the EBOV proteins (~250 kD), known as L. Similar to the L proteins of other members of *Mononegavirales*, it functions as the RNA-dependant RNA polymerase (RdRp) and exhibits a number of conserved properties. These include an above average leucine and isoleucine content, a large positive net charge (+57) at neutral pH, with clusters of basic amino acids interspersed with non-basic ones, as found in other RNA binding proteins (Muhlberger et al., 1992, Volchkov et al., 1999). Further, genome alignments showed that both the EBOV and MARV L proteins contain each of the three NNS virus polymerase motifs identified by Poch *et al.* (Poch et al., 1990). These include an RNA binding element

(motif A), a putative RNA template recognition and/or phosphodiester bond formation domain (motif B), and an ATP and/or purine ribonucleotide triphosphate-binding domain (motif C) (Poch et al., 1990). Additionally, highly conserved twin cysteine residues are present, as in all non-segmented, negative-sense, single-stranded virus L proteins, which may stabilize the secondary structures important for active site conformation (Poch et al., 1990).

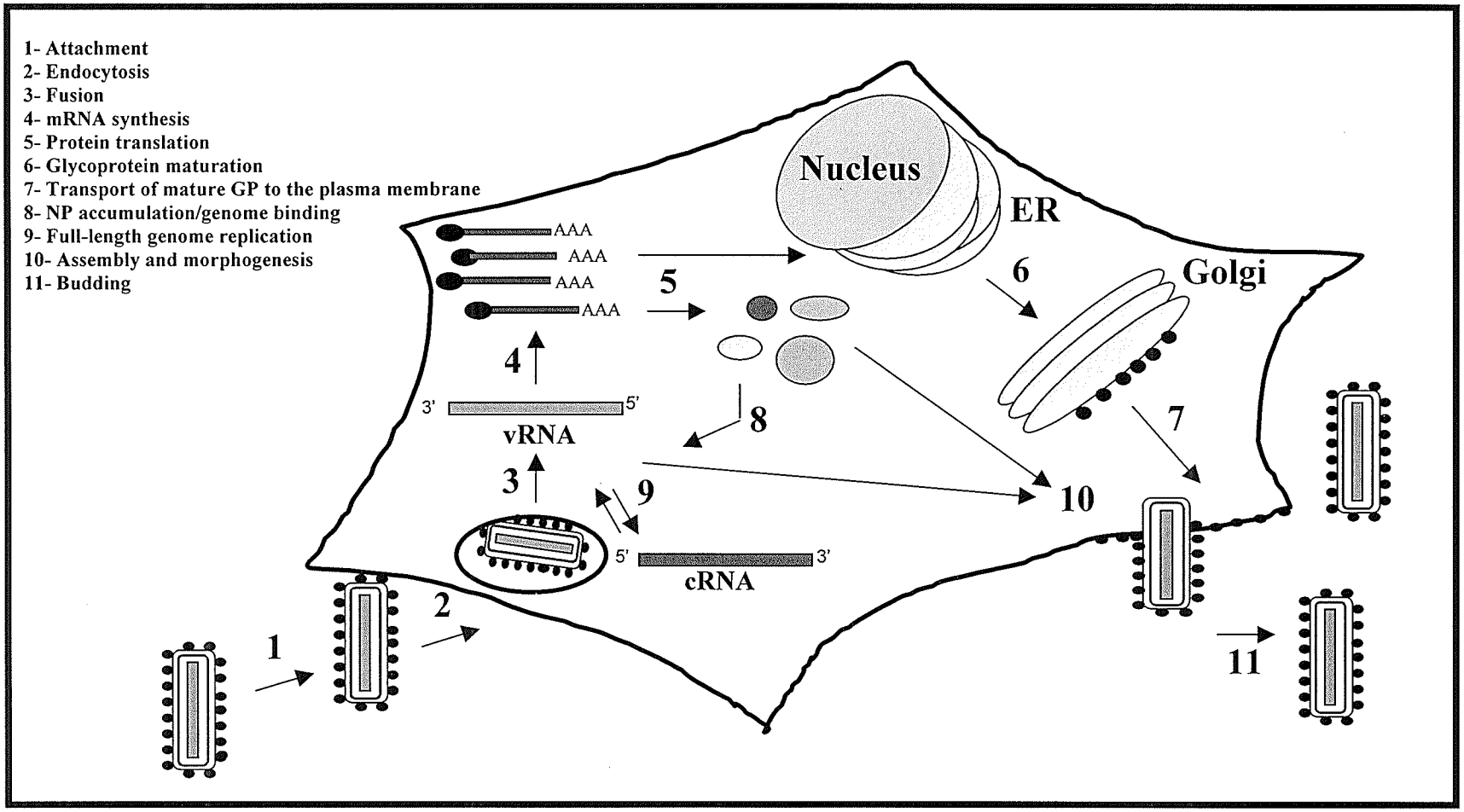
### 1.1.7 Viral Life Cycle

All the filoviruses are believed to share similar properties with respect to their life cycles, which can be broken down into the basic steps of (i) attachment and pH-dependent entry into the cell mediated by the surface glycoprotein; (ii) entry of the ribonucleoprotein (RNP) complex into the cytoplasm where transcription and replication take place; (iii) virus assembly and budding from the plasma membrane, a process driven by the matrix protein. Each of these phases of the life cycle is represented in Figure 6 and is discussed in detail below.

#### 1.1.7.1 Growth Characteristics

A number of cell types have been demonstrated to be susceptible to filovirus infection, which typically results in a lytic infection (Feldmann & Kiley, 1999). Interestingly, under some circumstances there are also indications that filoviruses may be capable of persistently infecting some cell lines (Strong & Feldmann, 2004), however, the molecular basis and significance of this observation is, as yet, unclear. Among the

**Figure 6. Overview of the viral life cycle.** The Ebola virus lifecycle begins with attachment to a cell surface receptor and endocytosis. Subsequent conformational changes due to low pH are presumed to mediate release of the RNP complex into the cytoplasm where transcription of monocistronic mRNAs and replication of progeny vRNA, through a cRNA intermediate, occur. Translation of the mRNAs by the host machinery yields the seven structural polypeptides, as well as the non-structural proteins sGP and Δ-peptide. Of the structural components, only GP is trafficked through the ER-Golgi pathway where it is glycosylated and proteolytically processed before being transited to the cell surface. Following RNP assembly in the cytoplasm, budding occurs either through multivesicular bodies, which subsequently fuse with the plasma membrane, or directly from the plasma membrane.



many cell lines susceptible to filovirus infection, VeroE6 (*Cercopithecus aethiops* adult kidney) cells are the most frequently used for virus propagation, as well as virus isolation (Feldmann & Kiley, 1999). Additionally, their ability to infect primary target cells, including monocyte/macrophages, and endothelial cells has been experimentally widely used, particularly for studies involving pathogenesis (Geisbert et al., 2003c, Stroher et al., 2001, Wahl-Jensen et al., 2005a). Despite their lytic life cycle, filoviruses can be difficult to detect using a standard plaque assay. Indeed, while there are indications that in some laboratories this method is successful using some isolates in specific cell types (T. Geisbert, personal communication) others report "spindle-like" plaques (L. Kolesnikova, personal communication) or a complete absence of reliable plaquing. These difficulties have led to the increasing use of a modified "immunoplaque" assay, which combines a plaque assay with detection of viral antigen by immunofluorescence assay (IFA). Further, the recent development of a GFP-expressing ZEBOV (Hoenen et al., 2006, Towner et al., 2005) circumvents even the need for this immunofluorescence detection step in some experimental applications, making it an extremely powerful tool.

### 1.1.7.2 Viral Attachment and Entry

Filoviral particles appear to enter host cells by pH-dependant receptor-mediated endocytosis, facilitated by interaction of the fusion peptide in $GP_2$ with the host cell membrane (Geisbert & Jahrling, 1995, Sanchez et al., 2001). However, many of the specifics of this process remain unclear. It has long been suspected that the surface glycoprotein $GP_{1,2}$, which is the only virion protein of the surface of particles, mediates binding and entry. This speculation is heavily supported by a number of experimental

observations including the neutralizing activity of a number of monoclonal antibodies targeting the GP, as well as the observation that creating recombinant viruses carrying this protein (Garbutt et al., 2004, Jones et al., 2005) affects the range of infectable cells as well as virus tropism. Further, VLPs which contain only VP40 and $GP_{1,2}$ are able to interact with susceptible cells types (Bosio et al., 2004, Swenson et al., 2005, Wahl-Jensen et al., 2005a, Wahl-Jensen et al., 2005b) making them excellent tools for molecular investigation into virus binding , as well as potential vaccines (Swenson et al., 2005).

To date several potential receptors have been identified for filoviruses. These include the folate receptor, which was identified by Chan and colleagues (Chan et al., 2000) as a potential receptor for filovirus infection, where its expression was sufficient to facilitate infection of Jurkat cells, which are normally non-susceptible to filovirus infection. However, other cell types which are susceptible to filovirus infection (i.e. macrophages) lack the folate receptor, while others possessing this putative receptor remain refractive to infection (Simmons et al., 2003). Similarly, the asialoglycoprotein receptor was shown to facilitate MARV entry into hepatocytes (Becker et al., 1995). However, the absence of this receptor on a number of other cell lines that are susceptible to filovirus infection has lead to the speculation that it may be a liver-specific filovirus receptor (Becker et al., 1995). Finally, two non-integrins, dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) and L-SIGN, a homologue found on some endothelial cells, as well as a recently identified human macrophage galactose- and N-acetylgalactosamine-specific C-type lectin (hMGL) have also been recently identified as contributing to infection (Alvarez et al., 2002, Takada et al., 2004). It is suggested that

these molecules may function as factors for virus attachment and serve to concentrate virus close to the cell membrane so that interactions with additional cofactors can take place.

### 1.1.7.3 Viral Transcription and Replication

Once fusion with the endosome takes place, the RNP complex is released into the cytoplasm. The cytoplasm is then the site of transcription and replication of the virus genome, as well as the expression of viral proteins, with the exception of the glycoproteins, which transit through the ER and Golgi apparatus to the cell surface. As with all negative-sense RNA virus genomes, the filovirus genomic RNA is not itself infectious but needs to be associated with the viral RNP complex proteins, NP, VP35, VP30 and L to start transcription of plus-sense monocistronic mRNAs used for viral protein synthesis.

These mRNAs have a poly(A) tail (Sanchez & Kiley, 1987), whose addition is directed by the conserved transcriptional stop signal (3'-UAAUUCUUUUUU-5), as well as a non-coded 5' terminal cap structure (Volchkov et al., 1999). The transcriptional start signals are also well conserved (3'- CUNCNUNUAAUU -5'), although not as strictly as the stop signals (Ikegami et al., 2001, Sanchez et al., 1993). While these signals were originally identified based on mRNA transcript sequencing (Sanchez et al., 1989), they can now be readily identified based on their adherence to these consensus sequences. The efficiency of transcription of the various viral mRNAs is believed to be influenced by gene order, with the formation of secondary structures within the intergenic sequences contributing to a gradient of transcript synthesis. However, this is based on analogy to

vesicular stomatitis virus (VSV) (Abraham & Banerjee, 1976, Ball et al., 1999, Iverson & Rose, 1981) and has never been directly shown for filoviruses. In addition, the presence of gene overlapping and/or duplicated termination sites in EBOV have also been postulated to play a role in the regulation of gene expression by affecting attenuation of transcription at the intergenic regions.

Replication, which involves the synthesis of an anti-sense positive RNA (cRNA) complementary to the genomic vRNA, is also mediated by the RNP complex proteins, although VP30 is not required for this process as it is for EBOV transcription (Muhlberger et al., 1999). This cRNA then serves as a template for synthesis of additional progeny vRNA genomes (Sanchez et al., 2001). The complementarity of the genome extremities on both the genomic and antigenomic strands suggests identical encapsidation sites, for binding by NP, as well as identical entry sites for the polymerase for both transcription and replication. Further, since the negative sense genomic RNA (vRNA) serves as the template for both transcription and replication, both of which are mediated by the viral RdRp, a mechanism must exist to regulate these two processes. A number of observations in the Rhabdoviruses, including the biphasic temporal expression of viral proteins and replication, as well as the binding of NP to newly synthesized vRNA and complementary (cRNA) transcripts, have led to the hypothesis that NP levels within the infected cells regulate the balance between transcription and replication (Whelan et al., 2004). This regulation may involve the masking of the intergenic regions, particularly with respect to secondary structure formation and/or the accessibility of termination signals. However, while filoviruses are widely believed to employ a similar mechanism this has never been directly shown.

### 1.1.7.4 Morphogenesis and Budding

As the viral infection progresses, the cytoplasm develops prominent inclusion bodies, which become highly structured as the virus proteins assemble into RNP complexes. Immunoelectron microscopy has shown that the matrix protein (VP40) and nucleoprotein (NP) accumulate in these inclusions and are closely associated during viral morphogenesis **(Kolesnikova, 2002)**. While direct interaction between these two proteins has not yet been published, coimmunoprecipitation data indicate that this interaction may occur through the N-terminal beta-sandwich domain of VP40 (T. Hoenen, personal communication). In addition, recent data using the infectious VLP (iVLP) system, which models virus morphogenesis and budding, indicate that VP24 may be important for the correct assembly of RNPs, particularly with respect to the incorporation of VP35 and VP30 (Hoenen et al., 2006). Once assembled these RNPs may bud through multivesicular bodies (MVBs) where they obtain their lipid membrane (Kolesnikova et al., 2004). Alternatively the MVBs may act as a platform for interaction of VP40 and GP, which then transit together to the plasma membrane and direct virus budding from this site (Kolesnikova et al., 2004). In either case, this budding process may be facilitated by the recruitment of ESCRT-1 complex proteins to the site(s) of budding by proline-rich domains in the matrix protein, as discussed earlier.

## 1.1.8 Ebola Hemorrhagic Fever

Since its initial isolation in 1976, Ebola virus has been identified as a causative agent of viral haemorrhagic fever (VHF). VHF generally describes a severe clinical syndrome in human and/or non-human primates that commonly starts as a "flu-like"

illness but quickly develops into a multi-organ syndrome including hepatic dysfunction, haemorrhages, capillary leakage, hypotension, and shock. However, the clinical picture of VHF caused by different virus infections varies slightly, even between very closely related agents (Table 4).

**Table 4. Clinical features of haemorrhagic fevers caused by filoviruses.**

| Filovirus | Distinguishing Clinical Features | Incubation Period | Mortality |
|---|---|---|---|
| *Zaire ebolavirus* | Fever, severe prostration, maculopapular rash, bleeding and disseminated intravascular coagulation common | 2-21 d | 59-90% |
| *Sudan ebolavirus* | Fever, severe prostration, maculopapular rash, bleeding and disseminated intravascular coagulation common | 2-21 d | 53-79% |
| *Reston ebolavirus* | Asymptomatic | ----- | ----- |
| *Ivory Coast ebolavirus* | Fever, acute diarrhoea and cutaneous eruptions, haematuria | ND | 0%* |
| *Lake Victoria marburgvirus* | Fever, myalgia, nonpruritic maculopapular rash, bleeding and disseminated intravascular coagulation common | 2-14 d | 23-88% |

* Based on only two non-fatal ICEBOV cases that have been documented

### 1.1.8.1 Clinical Presentation

Following an incubation period, which typically lasts between 5 and 14 days, infected individuals experience an abrupt onset of fever. Additional early symptoms are relatively non-specific and may include chills, muscle pain, nausea, vomiting, abdominal pain and/or diarrhoea. All Ebola hemorrhagic fever (EHF) patients will show impaired coagulation to some extent, which can manifest as conjunctival haemorrhage, bruising,

impaired clotting at venipuncture sites and/or the presence of blood in the urine or feces (Sureau, 1989). Swelling of the lymph nodes and kidneys, as well as necrosis of the liver, lymph organs, kidneys, testis and ovaries can also occur. In fatal cases, gross pathological changes include haemorrhagic diatheses into the skin, mucous membranes and visceral organs, as well as the lumen of the stomach and intestines. While approximately 50% of individuals develop a maculopapular rash on the trunk and shoulders, massive bleeding is fairly rare and is mainly restricted to the gastrointestinal tract (Fisher-Hoch et al., 1985). Severe nausea, vomiting and prostration as well as trachypnea, anuria and decreased body temperature all indicate impending shock and suggest a poor prognosis. Case fatality rates associated with EHF infection range from 50% to 90% (Geisbert & Jahrling, 2004, Sanchez et al., 2001, Schnittler & Feldmann, 2003) with death usually occurring between 6 and 9 days after the onset of symptoms. Among survivors, a protracted period of convalescence is typical and a number of sequelae have been reported including athralgia, uveitis, orchitis and psychosis (Rowe et al., 1999). Interestingly, the existence of asymptomatically infected individuals was also recently documented during the 1994 and 1996 Gabon outbreaks (Leroy et al., 2002, Leroy et al., 2000). Further, the development of asymptomatic infection was not related to genotypic changes in the infecting virus, but seemed to be linked to the development of a strong early inflammatory response (Leroy et al., 2002, Leroy et al., 2000).

#### 1.1.8.2 Transmission

All the agents responsible for causing VHFs have known or presumed animal reservoirs. Although a clear reservoir species for filoviruses has not yet been identified,

current evidence suggests a possible role for several species of fruit bats (*Hypsignathus monstrosus*, *Epomops franqueti* and *Myonycteris torquata*), which appear to be asymptomatically infection by EBOV (Leroy et al., 2005). Unfortunately, there is little knowledge regarding the initiation of virus outbreaks, since they are sporadic and typically occur in areas lacking in adequate health care infrastructure. As a result, outbreaks are often well underway or even waning before data gathering can be initiated. However, it has been clearly demonstrated that infection is primarily spread during outbreaks through contact with blood, secretions or tissues from patients (Dowell et al., 1999, Francesconi et al., 2003). In particular, nosocomial spread of Ebola virus is a significant risk with cases often resulting following injections with contaminated syringes (Sanchez et al., 2001). These percutaneous infections are associated with the shortest incubation periods and the highest risk of mortality (WHO, 1978). Another significant risk factor for the transmission of EBOV infection is the care of infected family members, particularly if there is direct contact to body fluids or the cadaver (Baron et al., 1983, Dowell et al., 1999, Francesconi et al., 2003). In contrast, airborne person-to-person transmission appears to be relatively rare, although it is the only plausible explanation in some instances (CDC, 2001, Roels et al., 1999). Experimental transmission in non-human primates has been shown to occur through mucosal exposure (Jaax et al., 1996) and it is thought to be possible in humans as a result of contact between contaminated hands and the mucosa or eyes, but this has never been directly shown (Colebunders & Borchert, 2000). However, this does not appear to be a major contributing mechanism since all epidemics to date have been successfully controlled using isolation techniques without specific airborne precautions.

During the course of infection the transmissibility of filoviruses increases and transmission seems to be very rare during incubation (Dowell et al., 1999), although a single case was documented in which contact with a patient hours before the onset of symptoms resulted in transmission (Dowell et al., 1999). However, following convalescence, virus can persist for a prolonged period in immunologically privileged sites (Rodriguez et al., 1999). In particular, EBOV has been isolated from seminal fluid for up to 82 days after onset of symptoms and can be detected by RT-PCR for up to 91 days (Rodriguez et al., 1999). This was despite clinical recovery and apparently normal immune function.

### 1.1.8.3 Diagnosis

As clinical microbiology and public health laboratories are not generally equipped for the diagnosis of VHF agents, it is necessary that samples are sent to one of the few designated laboratories capable of performing the required assays. However, mobile laboratory units have recently been added to assist patient management and surveillance efforts during epidemics (e.g., Ebola outbreaks in Gulu, Uganda, and Mbomo, Republic of Congo; Marburg outbreak in Angola).

Of the available techniques for the diagnosis of EHF, reverse transcriptase polymerase chain reaction (RT-PCR) and antigen capture ELISA are the most useful for making a diagnosis in an acute clinical setting. Serology (IgM capture ELISA and IgG ELISA) is useful for confirmation and surveillance, but negative serology is not exclusive. Similarly, confirmation by virus isolation should ideally be achieved although, as with serology, a negative finding is not exclusive. Further, the utility of virus isolation

as a diagnostic procedure is restricted by the time required for this procedure as well as by biosafety concerns. For non-outbreak surveillance, immunoperoxidase staining of formalin-fixed biopsies is also available (Zaki et al., 1999) and has several advantages including its simplicity and the lack of any need for enhanced biocontainment.

However, despite all the achievements in laboratory diagnostics, it should be kept in mind that the diagnosis of EHF will initially have to be based on clinical assessment. Of particular relevance will be a history of travel to endemic areas, (i.e. West or Central Africa) as well as any rural travel or contact with sick/dead primates or potential patients. The differential diagnosis will include the more common causes of febrile illness associated with travel (e.g. malaria, typhoid fever, dysentery), as well as other possible causes of haemorrhagic fever (e.g. Yellow fever, Lassa fever).

#### 1.1.8.4 Treatment

Treatment of Ebola virus infection is mainly supportive in nature and involves a combination of intravenous fluid replacement, the administration of analgesics and standard nursing measures. The maintenance of fluid and electrolyte balance, as well as circulatory volume and blood pressure, are essential. Additionally, mechanical ventilation, renal dialysis and/or anti-seizure therapy may be required, while intramuscular injections, non-steroidal anti-inflammatory and anticoagulant therapies are generally contraindicated. It is also important to note that treatment for other possible etiologic agents (e.g. agents of bacterial sepsis) should not be withheld while an EHF diagnosis is being confirmed. Early treatment of putative EHF cases may also include ribavirin, despite the lack of any efficacy against filovirus infection, against the

possibility of another etiological agent. Only once the final laboratory diagnosis of filovirus infection has been made should treatment be stopped. Finally, it should be noted that this form of supportive care, while the standard in developed nations, is generally not available in Central Africa where the vast majority of cases occur. This is likely a contributing factor in the high case fatality rates observed with filovirus infection.

There are currently no specific antiviral drugs approved for the treatment of EHF, however, a number of experimental approaches have been developed in recent years. Perhaps the most promising of these makes use of a recombinant nematode anticoagulant protein c2 (rNAPc2), which is currently in clinical trials for the treatment of acute coronary syndromes. Despite a general contraindication towards the use of anticoagulants in the treatment of haemorrhagic fever disease, because disseminated intravascular coagulation is a hallmark of filovirus infection and, more recently, over-expression of tissue factor has been shown to play a role in this process, the possibility of inhibiting this pathway was considered. As a result, it was found that administration as late as 24h post infection led to a 33% survival in a uniformly fatal EBOV infected macaque model (Geisbert et al., 2003a). In addition, the survival time in remaining animals was significantly prolonged indicating that, while this therapy may not be sufficient on its own, it could be a valuable tool in the treatment of filovirus infections, and potentially other haemorrhagic diseases that involve over-expression of pro-coagulant molecules.

For a further, detailed discussion of Ebola virus treatment the reader is referred to (Bray & Paragas, 2002) and (Feldmann et al., 2005).

### 1.1.8.5 Vaccines

Historically, the high level of biological containment required to work with filoviruses has been a major block in the development of new treatments or vaccines; furthermore, because of the virulence of the wild type viruses, live attenuated vaccine strains are unlikely to be a viable option for immunization. However, the development of molecular techniques, enabling the manipulation of RNA genomes (Neumann et al., 2002a, Volchkov et al., 2001), may result in the development of new, safer attenuated vaccine strategies. Additionally, the development of effective animal models, other than nonhuman primates, has helped overcome a significant barrier to vaccine testing (Bowen et al., 1980, Bray et al., 1998, Connolly et al., 1999, Ryabchikova et al., 1999).

The development of an EBOV vaccine has been the focus for a relatively large number of research teams (Table 5) because of the very high mortality, the high public profile of this virus and the availability of three animal models (nonhuman primates, guinea pig and mouse). Early attempts to produce inactivated vaccines for EBOV species were unsuccessful (reviewed in (Feldmann et al., 2003, Geisbert et al., 2002)). More recently, several vaccine strategies using recombinant viruses and/or DNA vaccination have been successful in protecting rodents from EBOV (Table 5); however, almost all were completely unsuccessful in protecting nonhuman primates (Geisbert et al., 2002). The first vaccine to have proven efficacy in nonhuman primates was a DNA prime/adenovirus boost approach (Sullivan et al., 2000); however, this protocol required months to provide protective immunity and, therefore, was limited in its utility. However, subsequent studies using only a single does of the recombinant adenovirus part

of the initial vaccine resulted in protection of the nonhuman primates from a high challenge dose (1500 $LD_{50}$) just 28 days after immunization (Sullivan et al., 2003).

**Table 5. Summary of filovirus vaccine platforms.**

| Delivery System | Viral Protein | Animal Model Tested (Survival) | References |
|---|---|---|---|
| Vaccinia Virus | GP | Guinea Pig (60%)<br>Cynomologous macaque (0%) | (Gilligan et al., 1997)<br>(Geisbert et al., 2002) |
| VEEV Replicon | VP24 | Mouse (62%) | (Wilson et al., 2001) |
| | VP30 | Mouse (50%) | (Wilson et al., 2001) |
| | VP35 | Mouse (39%) | (Wilson et al., 2001) |
| | VP40 | Mouse (53%) | (Pushko et al., 2000, Wilson et al., 2001) |
| | NP | Mouse (100%)<br>Cynomologous macaque (0%) | (Wilson et al., 2001)<br>(Geisbert et al., 2002) |
| | GP | Mouse (90%)<br>Guinea Pig (80%)<br>Cynomologous macaque (0%) | (Pushko et al., 2000)<br>(Pushko et al., 2000)<br>(Geisbert et al., 2002) |
| | GP + NP | Cynomologous macaque (0%) | (Geisbert et al., 2002) |
| DNA | NP | Mouse (70-80%)<br>Guinea Pig (63%) | (Vanderzanden et al., 1998)<br>(Xu et al., 1998) |
| | GP | Mouse (50-100%)<br>Guinea Pig (87%) | (Vanderzanden et al., 1998)<br>(Sullivan et al., 2000, Xu et al., 1998) |
| | sGP | Guinea Pig (73%) | (Xu et al., 1998) |
| | GP + NP | Guinea Pig (100%) | (Sullivan et al., 2000) |
| DNA + Adenovirus | GP + NP | Cynomologous macaque (100%) | (Sullivan et al., 2000) |
| Adenovirus | GP + NP | Cynomologous macaque (100%) | (Sullivan et al., 2003) |
| VLP | GP + VP40 | Mouse (100%) | (Warfield et al., 2003) |
| VSV | GP | Mouse (100%)<br>Cynomologous macaque (100%) | (Garbutt et al., 2004)<br>(Jones et al., 2005) |

[modified from (Feldmann et al., 2003)]

More recently, a new vaccine strategy using recombinant, vesicular stomatitis virus (VSV) has been successful in both rodent and nonhuman primate models of ZEBOV infection. These vectors have a complete deletion of the wild type VSV glycoprotein open reading frame, which is substituted by the full-length functional

glycoprotein of ZEBOV (Garbutt et al., 2004). These recombinant viruses have the tropism of ZEBOV but are attenuated *in vivo* and do not cause disease themselves, indicating that the GPs of HF viruses can be inserted into this vector system without increasing virulence. The VSV-ZEBOV recombinant vaccine has been shown to protect mice when given 30 minutes after challenge and is effective in mice when administered by the intranasal and oral routes (S. Jones, personal communication). In addition, both the MARV and EBOV VSV-based vaccines have been shown to protect non-human primates from infection with the relevant agent after 28 days when given intramuscularly (Jones et al., 2005). If successful vaccination though intranasal and/or oral routes, similar to those observed in mice, can be shown in non-human primates, there is real potential for rapid mass immunization using this vaccine platform.

### 1.1.9 Pathogenesis

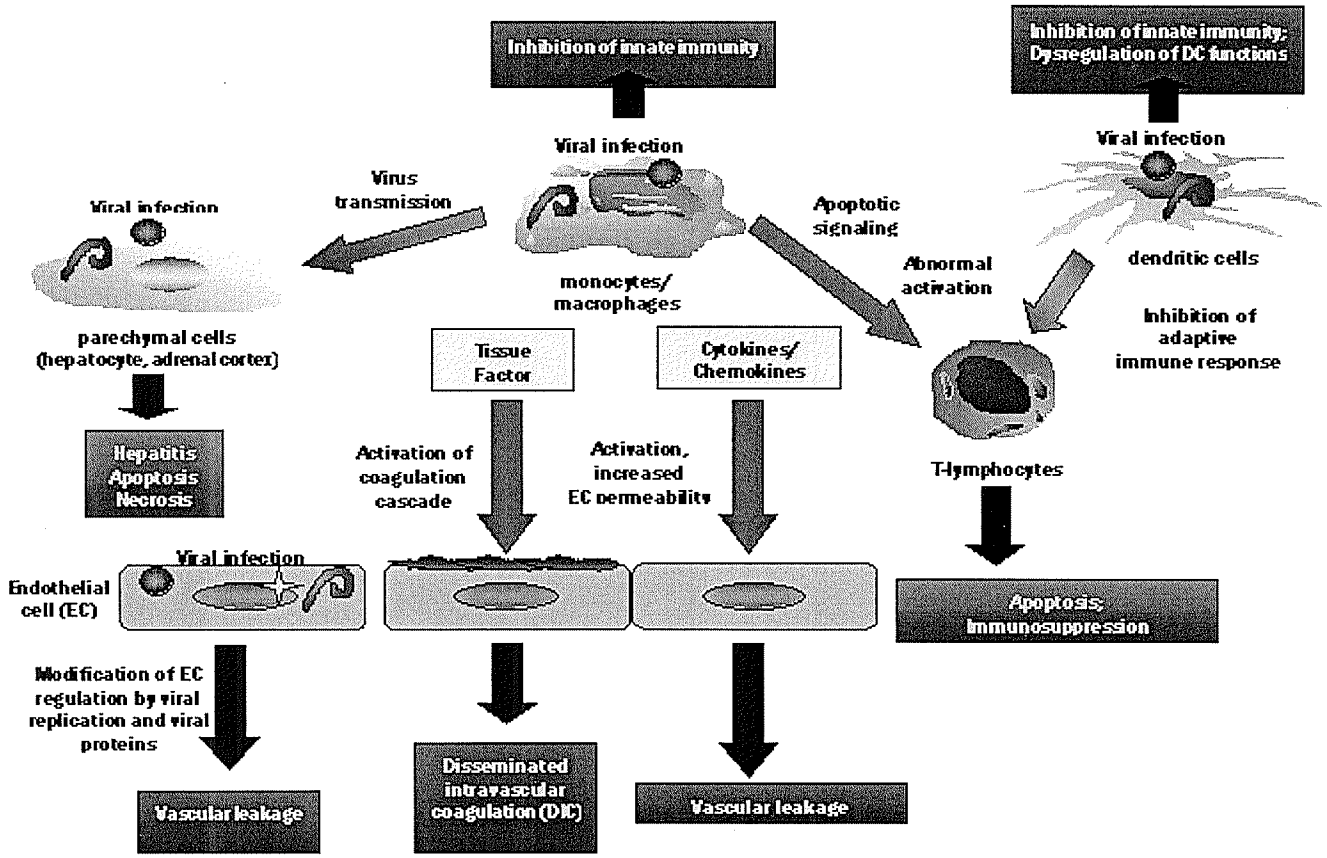
All filoviruses are associated with different degrees of virulence for human and/or nonhuman primates. MARV, ZEBOV and SEBOV, have caused several large VHF outbreaks in Africa, which were associated with high mortality rates. In contrast, only two confirmed cases of ICEBOV infection have ever been reported and there are no reports of VHF caused by REBOV (Sanchez et al., 2001).

The initial targets for filovirus replication are the mononuclear phagocytic cells (i.e. monocytes, macrophages) and dendritic cells (DCs) and from there infection spreads to fixed tissue macrophages in the liver, spleen and other organs (Schnittler & Feldmann, 1998, Zaki & Goldsmith, 1999). Subsequently, progeny virions infect hepatocytes, adrenal cortical cells, fibroblasts and, late in infection, endothelial cells. Visceral organ

necrosis, particularly in the liver, spleen and kidneys, is directly due to virus-induced cellular damage and this tissue destruction results in the exposure of underlying collagen and the release of tissue factor, which results in the development of disseminated intravascular coagulation (DIC) (Geisbert et al., 2003c). Abnormalities in blood coagulation and fibrinolysis are common during VHF infection and lead to bleeding manifestations (Figure 7). While in EHF, Marburg haemorrhagic fever and Crimean-Congo haemorrhagic fever, disseminated intravascular coagulation (DIC) is seen, DIC is not a prominent feature in other VHFs (Geisbert & Hensley, 2004, Peters, 1996, Sanchez et al., 2001, Schnittler & Feldmann, 2003). An important role of TF in triggering these abnormal coagulation cascades has been reported for EBOV infections in nonhuman primates (Geisbert et al., 2003c). Impairment of the microcirculation and the absence of inflammatory infiltration are also characteristic. Various degrees of lymphocyte depletion/apoptosis and necrosis in blood, spleen and lymphoid tissues without evidence of viral replication have been reported for several filoviral HF (Figure 7) (Geisbert & Hensley, 2004, Geisbert et al., 2000, Hensley et al., 2002). This seems to be further triggered by infected DCs, which fail to stimulate T-lymphocytes (Bosio et al., 2003, Mahanty et al., 2003).

Virus activation of primary target cells seems common and results in the systemic release of cytokines, chemokines and other mediators such as tissue factor (TF), which, in severe cases, might induce haemorrhages and a septic shock-like syndrome (Feldmann et al., 1996, Geisbert et al., 2003c, Schnittler & Feldmann, 1999, Schnittler & Feldmann, 2003, Stroher et al., 2001, Wahl-Jensen et al., 2005a). The virus-induced cytokine/chemokine profiles differ and may partly explain the distinct clinical pictures

**Figure 7. Summary of potential pathogenic mechanisms involved in filovirus infection** Early infection of monocytes/macrophages and dendritic cells suppresses host innate immune responses aiding in further dissemination. Further, the release of soluble factors from activated macrophages induces T-cell apoptosis as well as the activation of endothelial cells (ECs), which leads to increased vascular permeability. Over-expression of TF, also triggers coagulation dysregulation leading to disseminated intravascular coagulation (DIC), which can contribute to the development of multi-organ failure. Pathological endothelial changes are also caused by viral replication and viral protein-dependent pathways. In addition, necrosis of hepatocytes and adrenal cortical cells by viral replication leads to hypotension and shock. Red arrows indicate pathological changes during infection, green arrows indicate protective host responses. Grey boxes indicate mediators induced as a result of infection and red boxes indicate the end results of each pathological cascade.




[Altered from (Ebihara et al., 2005)]

observed with VHFs (Geisbert & Hensley, 2004). Infected macrophages also become activated and, thus, release a number of cytokines and chemokines that up-regulate cell surface adhesion and procoagulant molecules (Hensley et al., 2002, Stroher et al., 2001, Villinger et al., 1999). While these mediators seem to play a role in increasing endothelial permeability, destruction of endothelial cells (ECs) during infection is also suggested to contribute to the development of haemorrhagic diathesis and shock. However, in contrast to what was previously believed, it now appears that EC infection is secondary and only occurs in the terminal stage of infection (Bosio et al., 2003, Geisbert et al., 2003b, Geisbert et al., 2003d). In addition, virus- induced necrosis of hepatocytes and adrenal cortical cells furthers the development of hypotension and shock (Geisbert & Hensley, 2004). Further, both MARV and EBOV are capable of producing secreted glycoprotein products, which may further contribute to filovirus pathogenesis, although the mechanisms by which this might occur remain unclear (Schnittler & Feldmann, 2003).

## 1.2 Reverse Genetics Systems

### 1.2.1 Introduction to Reverse Genetics

Similar to the "classical" definition of to reverse genetics, in molecular virology this term describes the generation of systems in which mutations can be made and the resulting change in phenotype studied. Such systems can be broadly grouped into two categories: minigenome and infectious clone systems. Both utilize cloned cDNA to mediate either the expression of reporter genes (minigenome systems) or to produce infectious virus (infectious clone systems). Once generated, the genetic manipulation of

these systems (e.g. via point mutations, deletions, insertions, inversions, or translocations) using site-directed mutagenesis or PCR-based cloning strategies allows researchers to explore the effects of these changes on various stages of the virus lifecycle. Minigenome systems, which model virus replication and transcription, have proven to be excellent tools for studying these processes; and their application has already provided tremendous insights. In particular, these systems have, in many cases, been used to determined the proteins required for transcription and replication, as well as the ratios at which the proteins most efficiently carry out these functions (Conzelmann & Schnell, 1994, Durbin et al., 1997, Grosfeld et al., 1995, Kato et al., 1996, Muhlberger et al., 1998, Muhlberger et al., 1999, Radecke et al., 1995, Stillman et al., 1995, Yu et al., 1995), findings that have been instrumental in the subsequent establishment of infectious clone systems. While recent modification of the minigenome system to couple this process with VLP formation, yielding the infectious VLP system, has provided a tool to study viral entry and budding in a non-viral context (Hoenen et al., 2006, Watanabe et al., 2004), the *de novo* synthesis of RNA viruses from cloned cDNA via the infectious cDNA clone is perhaps the ultimate reverse genetics tool for studying the virus lifecycle (Hoenen et al., 2005, Neumann et al., 2005, Neumann et al., 2002a, Volchkov et al., 2001). The advent of this technique has provided researchers with a powerful tool to study, not only isolated single processes, such as virus replication or entry, but also the combined effects of a given factor on all viral processes and, ultimately, the net effect on viral pathogenesis. One further application of these systems is as a tool for screening antiviral drugs, as was recently shown for Lassa virus and Sendai virus (Hass et al., 2004, Pelet et al., 2005), although in the case of minigenome systems this is limited to

approaches that target transcription and replication. Finally, infectious clone systems can potentially be used in vaccine development, particularly for the production of attenuated viruses or the generation of virus recombinants.

### 1.2.2 History of Reverse Genetics

Infectious clone systems for positive-strand mammalian RNA viruses were established as early as 1981 (Racaniello & Baltimore, 1981). This early success was associated with the infectious nature of the viral RNA. Following delivery of the naked genomic RNA into eukaryotic cells, plus-strand viral RNA (vRNA) genome can act as mRNA to direct the synthesis of all the viral proteins necessary for subsequent stages in the virus life cycle, using the host cellular machinery. In contrast, negative-sense vRNA genomes are non-infectious because of their need to first transcribe their genomes into positive-sense mRNA prior to translation of viral proteins; a step that is dependent on the presence of the viral replicase complex, since eukaryotic cells lack the necessary enzymes to mediate (-) RNA → (+) RNA synthesis. Due to the additional complexity associated with establishing an infectious clone system for such negative-strand RNA viruses, the generation of a minigenome system has generally preceded the development of the infectious clone system, although this is not always the case. However, despite these added challenges, reverse genetics systems, have been successfully established for a number of negative-sense RNA viruses, including representatives of the families *Orthomyxoviridae*, *Bunyaviridae*, *Arenaviridae*, *Rhabdoviridae*, *Paramyxoviridae*, *Filoviridae* and *Bornaviridae* over the past decade (reviewed in (Neumann et al., 2002b)). In each instance the technology used varies to reflect both the particular

requirements of the virus as well as the availability of established methodologies at the time of construction.

The first development of an artificial vRNA analogue, or "minigenome", from cloned cDNA was achieved for the paramyxovirus, Sendai virus (Park et al., 1991). These vRNA analogues, which are miniaturized mimics of the authentic viral genome, consisted of a cDNA encoding a reporter protein, chloramphenicol acetyltransferase (CAT), flanked by the minimal regulatory sequences at the genome ends (i.e., the non-coding region [NCR] sequences) under the transcriptional control of the bacteriophage T7 RNA polymerase (T7) promoter. However, transcription and replication of this minigenome still required infection with Sendai virus. The first entirely plasmid-based minigenome approach was developed for vesicular stomatitis virus (VSV) by Pattnaik et al. and relied on the expression of the RNP proteins from multiple plasmids, whose transcription was driven by the T7 promoter (Pattnaik et al., 1992) following infection with a recombinant vaccinia virus expressing the T7 RNA polymerase (vTF7-3) (Fuerst et al., 1986). This system provided T7 in the cytoplasm and, thus, avoided potential issues with nuclear processes such as splicing and the export of transcripts. However, one potential limitation of the use of T7 to mediate minigenome RNA transcription is that it displays variability with respect to the exact site of transcription initiation, which can lead to the production of RNAs that contain extra non-coded nucleotides at the genome ends. Since the terminal regions of many viral genomes form secondary structures through base-pairing that are believed to be important for their function, such additional nucleotides may have potentially deleterious effects if they perturb the formation of these structures. Therefore, in order to provide authentic 3' ends to the viral minigenome RNA

a hepatitis delta virus (HDV) ribozyme sequence was inserted at the 3' end of the minigenome cassette (Pattnaik et al., 1992). In this way, the autocatalytic cleavage properties of the HDV ribozyme were exploited to generate the exact viral 3' ends.

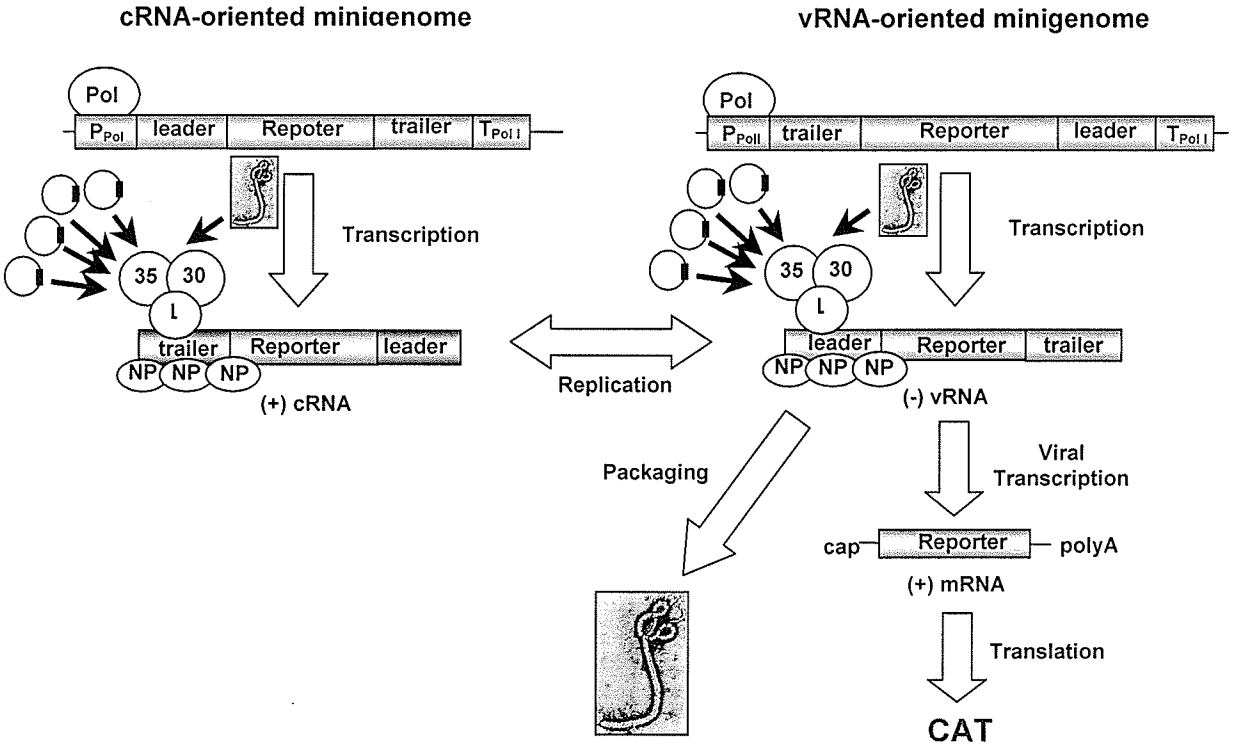
It was not until the generation of recombinant rabies virus by Conzelmann and colleagues (Schnell et al., 1994) that it was demonstrated, for the first time, that rescue of a non-segmented negative-sense single-stranded RNA virus entirely from cDNA was possible. In this system, a full-length positive-sense cDNA copy of the of rabies virus genome and the viral replicase complex components, consisting of the viral nucleocapsid protein (N), polymerase cofactor protein (P), and RdRp (L), were produced from plasmids under the control of the T7 promoter. These plasmids were transfected into eukaryotic cells and the T7 polymerase was provided by infection with vTF7-3 with correct 3' genome ends generated by HDV ribozyme cleavage. Providing anti-genomic (positive-sense) RNA transcripts was found to be the critical step for the success of this system because it minimized hybridization of N, P, and L mRNAs to the naked negative-sense genomic RNA. Therefore, this study was instrumental in initiating the development of other reverse genetics systems for members of *Mononegavirales*, including other members of *Rhabdoviridae* (Conzelmann, 1996, Conzelmann, 1998, Conzelmann & Meyers, 1996, Lawson et al., 1995, Roberts & Rose, 1998) as well as *Paramyxoviridae* (Baron & Barrett, 1997, Collins et al., 1995, Garcin et al., 1995, He et al., 1997, Parks et al., 1999) that were based on T7 polymerase supplied by infection with 'modified vaccinia virus Ankara' (MVA-T7). However, this system has the disadvantage of requiring separation of the recombinant viruses of interest from progeny of the recombinant vaccinia virus. Therefore, alternative methods of providing the T7 RNA

polymerase have also been developed and include the use of plasmids expressing the protein from eukaryotic promoters (Lee et al., 2002, Neumann et al., 2002a) or expression of the polymerase from a stably transfected cell line (Buchholz et al., 1999, Radecke et al., 1995, Volchkov et al., 2001).

### 1.2.3 Minigenome Systems for Filoviruses

In the case of filoviruses, minigenome systems for MARV (strain Musoke) and ZEBOV (strain Mayinga) were developed in 1998 and 1999, respectively, using the T7 RNA polymerase to synthesize negative-sense vRNA transcripts from cDNA (Muhlberger et al., 1998, Muhlberger et al., 1999). Initially, the cDNA constructs contained the 3' leader, the non-coding region of the NP gene, the 5' non-coding region of the L gene and the 5' trailer sequences of the genome flanking the single reporter gene CAT (Figure 8), although in recent years additional reporter systems have been used. These minigenome systems were driven either by a helper virus infection or transfection of plasmid DNA encoding the RNP complex proteins to provide the necessary machinery for transcription and replication of the minigenome construct (Figure 8). In the past 5 years, both systems have allowed the study of different aspects of filovirus transcription and replication. In particular, using this minigenome system it was established that only three of the four- nucleocapsid proteins, NP, VP35 and L, were necessary to support replication and transcription of the monocistronic MARV minigenomes (Muhlberger et al., 1998). This is in agreement with data obtained from various paramyxovirus and rhabdovirus systems where the nucleoprotein (N), phosphoprotein (P) and the RNA-dependent RNA polymerase (L) are the minimum proteins required for replication

**Figure 8. Transcription and replication steps in a minigenome system.** Cells are transfected with a minigenome plasmid containing a reporter gene flanked by the genomic leader and trailer regions under control of either a T7 or a Pol I promoter. Transcription of an initial vRNA-like molecule is mediated by these polymerases. Subsequent transcription and replication of the vRNA-like molecule can be driven either by helper plasmid encoded ribonucleoprotein (RNP) complex components (nucleoprotein [NP], virion protein [VP] 35, VP30, RNA-dependent RNA polymerase [L]) or by infection with live virus (helper virus). Transcription of reporter mRNA transcripts from the vRNA-like minigenomes by the RNP complex proteins and subsequent translation leads to expression of the reporter gene.




**[Altered from (Groseth et al., 2005)]**

(Conzelmann & Schnell, 1994, Durbin et al., 1997, Grosfeld et al., 1995, Kato et al., 1996, Radecke et al., 1995, Stillman et al., 1995). However, using the minigenome system for ZEBOV, which was generated using the same strategy as for the MARV system, it was shown that all four of the nucleocapsid proteins NP, VP35, VP30 and L were required for efficient replication and transcription of monocistronic minigenomes (Muhlberger et al., 1999). This reconstituted minigenome system was also used to show that okadaic acid, which is an inhibitor of PPI and PP2A, could block ZEBOV specific

transcripts production through the dephosphorylation of VP30 (Modrof et al., 2002). More recent studies with this system have further identified the role of VP30 as an early anti-termination factor, by facilitating the analysis of mRNA products generated in the absence of VP30 as well as by allowing mutations in the hairpin region of the 3' non-coding region of the genome, which VP30 is believed to resolve, in order to eliminate the requirement for VP30 (Weik et al., 2002).

### 1.2.4 Infectious VLP Systems for Filoviruses

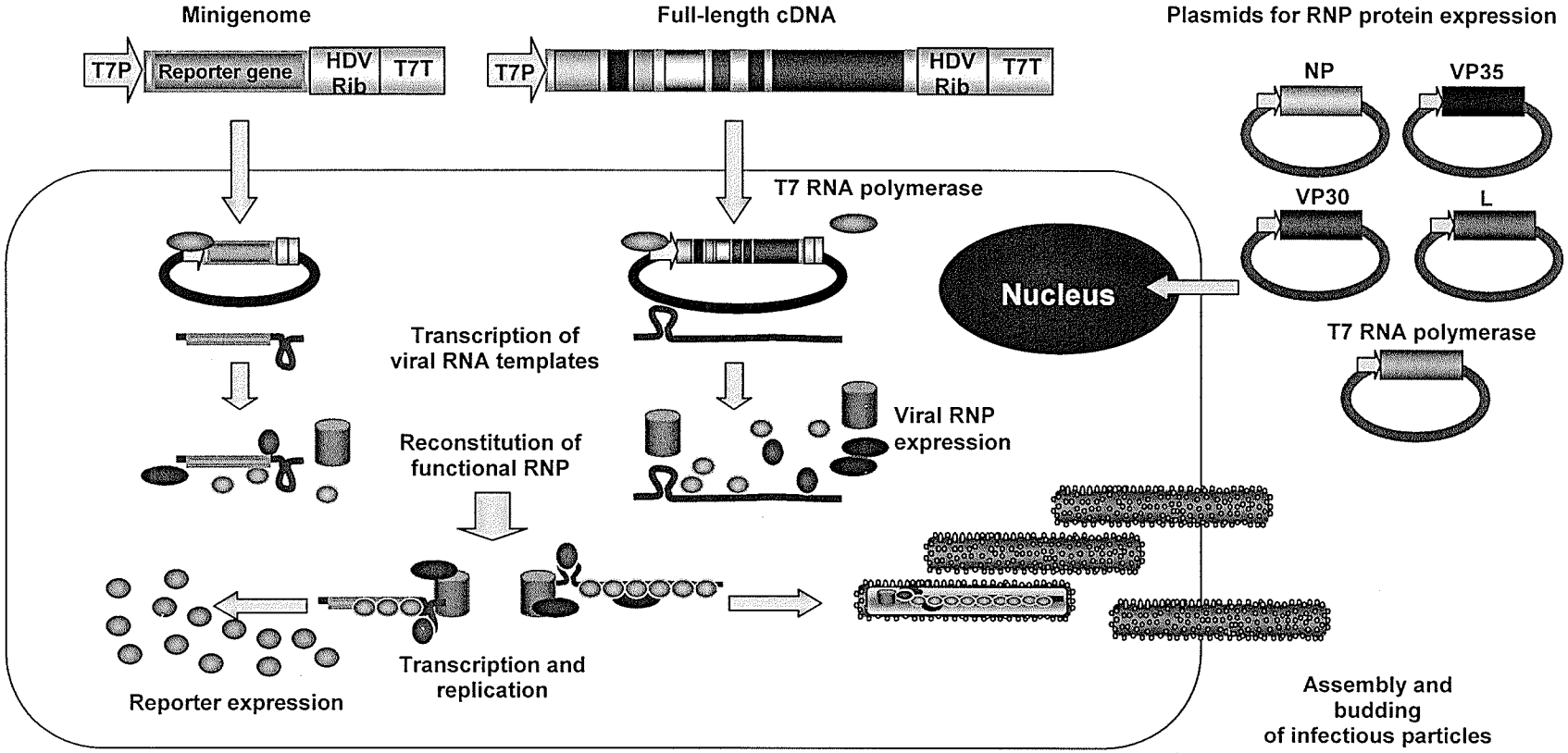
A recent modification of the minigenome system, in which it is coupled to VLP formation, is the infectious VLP system. Variations of this approach have been reported for a number of other viruses, including *Vesiculovirus, Influenzavirus A*, *Bornavirus*, *Lymphocytic choriomeningitis virus* and *Thogotovirus* (Lee et al., 2002, Neumann et al., 2000, Perez & de la Torre, 2005, Stillman et al., 1995, Wagner et al., 2000, Whelan et al., 1995), however, it was first developed for filoviruses by Watanabe *et al.* (Watanabe et al., 2004) at which time it was used to identify the minimal requirements for RNP incorporation into virions. While still a relatively new system, other groups are now developing this system further to study the role of the individual proteins and/or their oligomeric states in the virus life cycle, as well as for use a tool to screen potential antiviral approaches which target a variety of steps in the virus lifecycle (T. Hoenen, personal communication), under biosafety level 2 (BSL2) conditions.

### 1.2.5 Infectious Clone Systems for Filoviruses

As noted earlier, minigenome systems are the building blocks of an infectious clone. These systems utilize the same principles as a minigenome system, but rather than a reporter gene being expressed by the nucleocapsid proteins, the full viral genome is replicated and transcribed, producing infectious, fully functional viruses (Figure 9). The first such infectious clone system for a filovirus was developed for *Zaire ebolavirus* by Volchkov et al. in 2001 (Volchkov et al., 2001) and was based on transfection of T7-driven plasmids encoding the genome as well as NP, VP35, VP30 and L into BSR T7/5 cells, which stably expressed the T7 RNA polymerase needed to synthesize both the viral proteins and genome transcript (Figure 9). Shortly thereafter, Neumann and colleagues (Neumann et al., 2002a) demonstrated that T7 could also be supplied via an additional plasmid, which encoded the T7 polymerase under the control of a eukaryotic promoter. In this system a chicken β-actin-derived RNA polymerase II promoter was used to facilitate expression of both the RNP proteins, as well as the T7 RNA polymerase, while the full-length genome plasmid was under T7 promoter control (Figure 9). Interestingly, both positive-sense and negative-sense oriented full-length genomic RNA clones allowed for the rescue of infectious virus in this system (Neumann et al., 2002a).

Using the infectious clone system, a limited number of studies have been published either addressing the function of selected virus proteins or describing the expression of a foreign gene (i.e. green fluorescent protein [GFP]) from an additional transcription unit (Hoenen et al., 2005, Neumann et al., 2005, Neumann et al., 2002a, Towner et al., 2005, Volchkov et al., 2001) (H, Ebihara personal communication; S, Theriault personal communication). In particular, the glycoprotein, which is though to be

**Figure 9. *Zaire ebolavirus* reverse genetics systems.** This scheme illustrates the components of the system and the steps involved in the rescue of infectious virus using the infectious clone system and those involved in the rescue of reporter activity from the minigenome system which models the majority of these steps. In both cases transfection of RNP protein encoding plasmids leads to synthesis of NP, VP35, VP30 and L. Cotransfection of either a full-length genome encoding plasmid or a minigenome non-coding plasmid leads to the production of the relevant transcript, both of which can be recognized by the viral RNP proteins. Subsequent transcription leads in the case of the minigenome system to the production of assayable reporter protein, while in the infectious clone system viral genome copies are produced, along with the remaining viral protein, including those necessary to mediate budding of new virus particles.




**[altered from (Ebihara et al., 2005)]**

a major determinant for pathogenesis, has been studied using these systems. Volchkov and colleagues (Volchkov et al., 2001) investigated the importance of the editing site within the glycoprotein of EBOV. Using site directed mutagenesis, the seven adenosine editing site was interrupted by adding two guanidines (i.e. AAGAAGAA) and an additional adenosine to keep the defined open reading frame, such that only $GP_{1,2}$ was produced (Volchkov et al., 2001). Using this approach it was demonstrated that without editing effective replication and transcription were unhindered. However, the increased expression of full-length glycoprotein resulted in reduced virus particle release due to stronger cytopathic effects, including increased cytolysis. It was demonstrated that glycoprotein synthesis was of an immature precursor with high-mannose type sugars, indicating that glycoprotein transport was largely arrested in the endoplasmic reticulum or in an early Golgi compartment (Volchkov et al., 2001), indicating that over-expression of the glycoprotein may lead to cell death by exhausting the processing machinery of the cells. On the other hand, there is evidence that $GP_{1,2}$ itself displays cytotoxicity, which seems to be associated with the mucin-like domain found in $GP_1$ (Sullivan et al., 2005, Yang et al., 2000). However, cytotoxicity depends on the level of glycoprotein expression and, thus, expression of sGP (non-edited transcript) seems to control the cytotoxicity associated with transmembrane glycoprotein $GP_{1,2}$, expression and, therefore, lead to enhanced virus load and spread in the infected host (Volchkov et al., 2001).

In order to establish the significance of EBOV glycoprotein cleavage, which for many other viruses is known to be a prerequisite for fusion between the viral envelope and cellular membranes and, therefore, is an important step in pathogenesis (Klenk & Garten, 1994), a cleavage-deficient ZEBOV mutant with an altered furin cleavage motif

in pre-GP was generated by Neumann and colleagues (Neumann et al., 2002a). Although pre-GP cleavage was undetectable, the mutant virus grew to titres only slightly lower than that of wild-type virus indicating that, while cleavage may enhance infectivity, it is not crucial for *in vitro* infectivity (Neumann et al., 2002a). One possible explanation for this finding is that the EBOV fusion peptide has an unusual location 28 amino acids from the amino-terminal end of $GP_2$ (Ito et al., 1999, Sanchez et al., 1998). It is also flanked by two cysteine residues) which are thought to form a disulfide bridge, thus, exposing the fusion peptide in the form of a loop (Feldmann et al., 2001, Gallaher, 1996). This unusual localization and structure might allow sufficient exposure of the fusion peptide to interact with the cellular membrane, following a low-pH shift, even if GP remains uncleaved and thus, could explain that infectivity is largely independent of cleavage. Recent data from animal studies further indicated that the cleavage of GP has no influence on the pathogenicity of EBOV *in vivo* (T. Geisbert, personal communication).

## 1.3 RNA Interference

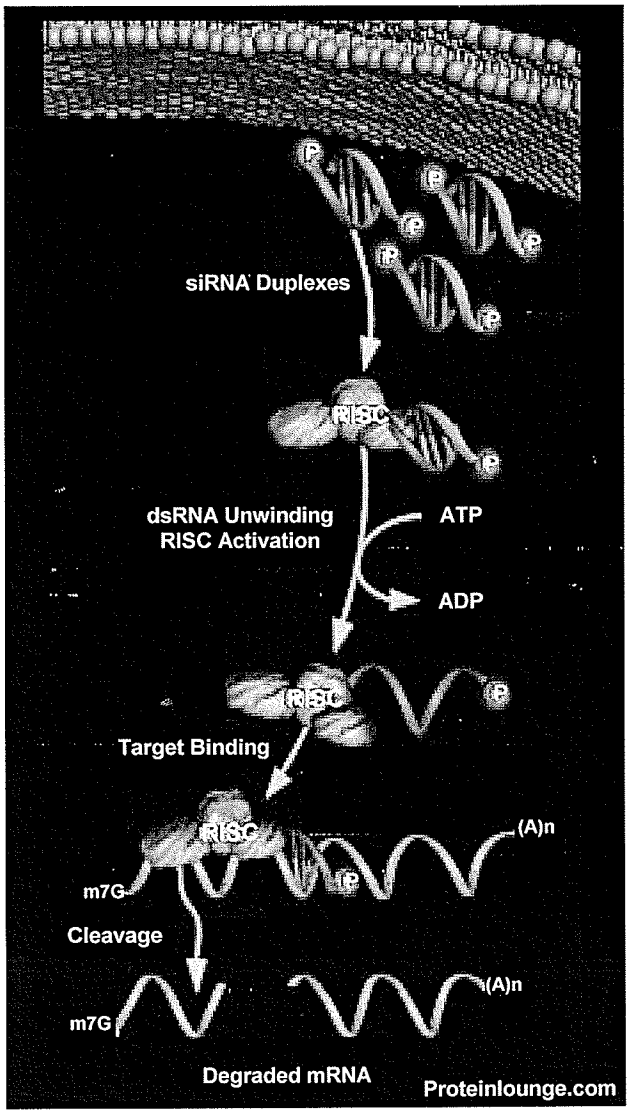
RNA interference (RNAi) is a common feature of the posttranscriptional gene silencing (PTGS) mechanisms observed in a variety of organisms including plants, invertebrates, fungi and animals (Elbashir et al., 2001a, Fire et al., 1998, Jorgensen, 1990, Romano & Macino, 1992). The natural role of RNAi is to act as a defence mechanism to protect the genome against undesirable RNAs, such as those from RNA viruses and mobile genetic elements and aberrant transcription products (Baulcombe, 2004, Gitlin & Andino, 2003, Plasterk, 2002, Voinnet, 2001). However, it has more recently become known for its potential as a scientific research tool (Dykxhoorn et al., 2003) and for use

as an antiviral therapy (Gitlin & Andino, 2003), where it has been shown to be a powerful tool for experimentally reducing the expression of specific genes.

### 1.3.1 Introduction to RNA Interference

The mechanism of RNA interference (RNAi) is triggered by double-stranded RNA (dsRNA) and leads to the silencing of gene expression (Fire et al., 1998) (Figure 10). In its simplest form this process uses the introduction of long (i.e. typically >200 nt) dsRNAs into cells to facilitate the degradation of mRNAs in a sequence-dependent manner (Hammond et al., 2001). Once these long dsRNAs are introduced into cells, both an initiator and an effector step are required before they are active in PTGS (Hutvagner & Zamore, 2002), the pathway for which is shown in Figure 10. During initiation, Dicer, a member of the RNase III family of ribonucleases, binds to the dsRNA and cleaves it into small interfering RNA (siRNA) duplexes (Bass, 2000, Bernstein et al., 2001, Dykxhoorn et al., 2003), which are composed of 21-23 base pair dsRNA duplexes with 2-nt 3' overhangs, a 5'-monophosphate and a 3'- hydroxyl group (Elbashir et al., 2001a, Elbashir et al., 2001b). In the effector step, the siRNAs are incorporated into RNA induced silencing complexes (RISC), a process which is dependant on the 5'-terminal phosphorylation of the siRNA (Khvorova et al., 2003). The helicase domain of RISC binds to one end of the duplex and unwinds the double- strand in an ATP-dependent manner with the thermodynamic stability of the first few base pairs affecting the ratio of antisense to sense strands of siRNA incorporated (Dorsett & Tuschl, 2004). These activated RISCs then scan homologous transcripts through base pairing interactions and,

**Figure 10. RNA induced silencing pathways in mammalian cells.** Following entry into the host cell, long dsRNAs (>300 bp) trigger the IFN response, however, shorter dsRNAs (<30bp) are able to interact with the RNA-induced silencing complex (RISC) where the strands are unwound and presented. These ssRNAs in the context of a RISC complex can then bind to target mRNAs. The resulting double stranded regions trigger cleavage of the mRNA by RISC after which the RISC-RNA complex is recycled for further targeting.




when a completely complementary match is found, cleave the targeted mRNAs at the site corresponding to the $10^{th}$ and $11^{th}$ nucleotides from the 5' terminus of the siRNAs (Ahlquist, 2002, Khvorova et al., 2003). Within the RISC complex, it is an Argonaute family member in contact with the single-strand siRNA that is believed to mediate this

cleavage event through its RNaseH-like domain (Liu et al., 2004, Song et al., 2003, Song et al., 2004).

While this approach to RNAi was initially proven to function as a method of knocking down gene activity in invertebrates such as *D. melanogaster* and *C. elegans* (Gura, 2000), in most mammalian cells the introduction of long dsRNA (>30 nt) initiates a cellular IFN response, leading to non-specific inhibition of protein synthesis and RNA degradation, host cell shutdown and apoptosis (Gil & Esteban, 2000), either through activation of RNA-activated protein kinase (PKR) (Manche et al., 1992) or RNaseL (Minks et al., 1979). While virus infection produces dsRNA, viruses often avoid inducing a profound cellular response by the use of RNA-binding proteins that block access to the RNA, thereby avoiding the IFN response as well as RNAi directed against the genome (Haller et al., 2006). Recently, it has become clear that synthetic small siRNAs can directly interact with the RNA-induced silencing complex (RISC) to cleave target mRNA (Dykxhoorn et al., 2003) and that, due to their small size, these sequence-specific 21-23 nucleotide RNA duplexes can initiate PTGS without triggering immunological effects with the resulting silencing typically lasting for 3-4 days before mRNA recovery begins (Semizarov et al., 2003). It is this siRNA-based approach that is now being widely employed to silence gene expression in mammalian and, in particular, human cells.

This technology has previously been employed to manipulate gene expression, to study signalling pathways and to identify gene functions (Gura, 2000). In addition to these basic research applications, RNAi is now being increasingly developed in the hopes

of preventing and/or treating human diseases (Lu et al., 2005, Sioud & Iversen, 2005, Tong et al., 2005). Despite significant advancements in the treatment of virus infections, the utility of many of the current drugs is restricted by factors such as toxicity, cost and the acquisition of resistance (Dave & Pomerantz, 2003). Thus there is substantial interest in the application of siRNA technology to this field, with results thus far having shown the ability to protect host cells from viral infection, inhibit the expression of viral antigens, decrease transcription and/or replication of viral genome, impair the assembly of viral particles and influence virus-host interactions (reviewed in (Tan & Yin, 2004)).

### 1.3.2 siRNA Design

The ability of siRNAs to mediate RNA silencing, and the efficiency at which they do so, are difficult to predict and are still not fully understood. While there are an ever-increasing number of known sequence requirements, even sequences based on these rules must be verified experimentally. In fact, it is still often necessary to test a number of potential targets in order to identify one that is sufficiently potent. Of the known parameters, one of particular importance is the thermodynamic stability of the 5' antisense end of the duplex. Analyses of the stability of published siRNA sequences indicated that functional duplexes displayed lower internal stability in this region relative to the rest of the sequence than non-functional duplexes (Khvorova et al., 2003, Schwarz et al., 2003). The size of the siRNA is also of critical importance, with dsRNAs < 30 bp being used to avoid non-specific silencing through the IFN response, as mentioned above. Additional recommendations include avoiding the first 50-100 nucleotides downstream of the start codon and the 100 nucleotides located upstream of the stop codon, as well as

the 5' and 3' UTRs, as these regions may contain binding sequences for regulatory proteins that may affect the accessibility of the RNA target sequence to the RISC complex. However, this is not an absolute requirement since several genes have been targeted using these regions (McManus et al., 2002, Rubinson et al., 2003, Yu et al., 2002). Similarly, low GC content sequences are usually recommended, although there are examples of functional siRNA sequences that deviate from this rule as well (Bertrand et al., 2002, Hasuwa et al., 2002). When using a small hairpin RNA (shRNA) system there are also several additional considerations, including the starting nucleotide and the size of the loop region. In particular, if an RNA Pol III promoter is used to mediate transcription of the shRNA, as is usually done (Brummelkamp et al., 2002), the first nucleotide of the siRNA sequence should be chosen to be an A or a G, since transcription is more efficient when the first transcribed nucleotide is a purine (Myslinski et al., 2001, Paddison et al., 2002). In addition, the loop size for the shRNA should be between 5-9 nt in length in order to produce the most effective constructs (Paddison et al., 2002).

While well-designed siRNAs will result in a strong and specific silencing response it is important to control off-target effects, which have been reported by a number of groups (reviewed in (Hannon & Rossi, 2004)). One source for these effects is activation of the IFN-response through PKR. Although this mechanism is thought to be principally stimulated by dsRNAs >30bp it has been reported that siRNS/shRNAs can, under some circumstances and in certain cell types, result in generalized RNA degradation and repression of protein synthesis through this pathway (Bridge et al., 2003, Persengiev et al., 2004, Sledz et al., 2003). However, screening a sequence to ensure that it has less than 15 adjoining homologous nucleotides to other sequence in the genome in

the species of interest can usually minimize these effects. In addition, examination of a secondary structure of the mRNA target can help eliminate possible sequences with poor accessibility and can, therefore, improve the chances of selecting a potent target site.

### 1.3.3 Alternative siRNA Generation

While chemically synthesized siRNAs can induce potent gene silencing, they are expensive and induce only transient gene silencing due to their short lifetime in the cell. Typically, silencing using this approach lasts for only 3-4 days before expression begins to recover. Therefore, to obtain gene silencing for prolonged periods of time, systems of intracellular siRNA expression have been developed. Among these is the use of shRNAs, in which a sense and antisense strand are expressed separated by a non-specific loop sequence. These constructs depend on their interaction with Dicer to process them into authentic siRNA structures that can function in the RNA interference pathway (Lee et al., 2004). Usually the transcription of these constructs is driven from H1 or U6 RNA Pol III promoters (Brummelkamp et al., 2002, Paddison et al., 2002), which drive transcription of the corresponding small nuclear RNAs. These promoters are often chosen because of their relatively compact structure, however, both RNA Pol I and RNA Pol II promoters have been successfully used as well (McCown et al., 2003, Xia et al., 2002). The shRNAs that result from this method are more stable than synthetic siRNAs and are continuously expressed within the cells. Both these features contribute to a longer-lasting silencing effect. However, one possible limitation with this approach *in vivo* is the ability of bacterially derived plasmid DNA to induce an IFN-response through

toll-like receptor (TLR) –9 signalling (Knapp et al., 2002, Krieg, 2002, Takeshita et al., 2004).

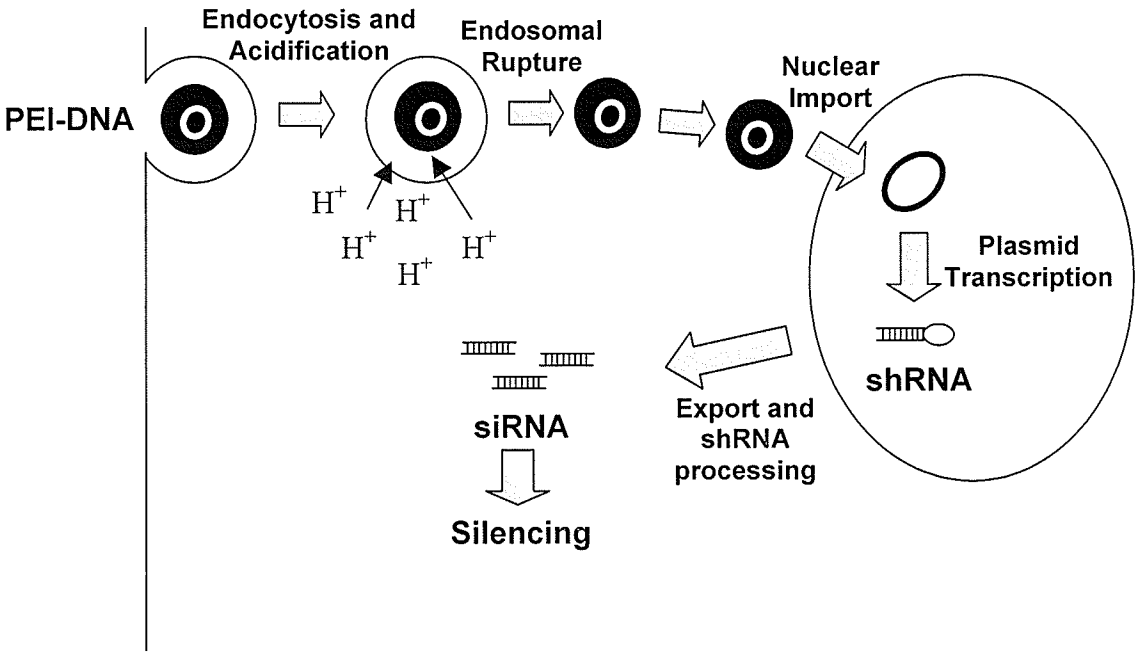
Another approach for generating siRNAs is to cleave long dsRNAs using bacterial RNase III (Yang et al., 2002, Yang et al., 2004). This generates a mixed pool that theoretically contains all possible 20-25bp siRNA sequences within a target region. This is not only an inexpensive method of producing siRNAs, but has the potential to decrease the formation of escape mutants. Additionally, this approach eliminates concerns regarding the efficiency of targeting for a given siRNA sequence.

### 1.3.4 siRNA Delivery Using Polyethylenimines

While the effects of RNAi *in vitro* have been very clearly demonstrated, a major stumbling block to their application *in vivo*, is the issue of delivery. One very promising approach is the development of linear polyethylenimines (PEIs) as delivery vehicles for DNA and siRNAs. Upon mixing, these transfection reagents condense DNA into ~ 50 nm positively charged particles, which are small enough to diffuse into animal tissues. These particles are then able to interact with ubiquitously expressed anionic proteoglycans and are taken up by endocytosis (Kopatz et al., 2004) (Figure 11). One of the unique properties of PEIs is that within the endosome they act as "proton sponges", using nitrogen atoms available for protonation to buffer changes in pH (Boussif et al., 1995). In addition to protecting the encapsidated DNA, this leads to endosomal swelling and rupture (Akinc et al., 2005), which facilitates release of the DNA into the cytoplasm (Figure 11). Finally, unlike branched PEIs or cationic liposomes, linear PEIs can

enhance entry into the nucleus across the nuclear membrane, although the mechanism by which this occurs is unclear (Brunner et al., 2002). This is particularly valuable for the delivery of plasmid DNAs, which must first access the nucleus prior to transcription by host polymerases. As such, these PEI-based approaches can be applied to the delivery of both shRNA expression plasmids and siRNA molecules. This feature should also allow the direct comparison of shRNA strategies against conventional, chemically synthesized siRNAs, in future.

**Figure 11. Mode of action of PEI delivered nucleic acids.** Delivery of DNA into the cell is facilitated by endocytosis of the complexes, either through interaction of the positively charged nitrogen residues in the PEI with various anionic surface proteoglycans, or through specific interaction of coupled components, such as mannose or galactose, with their receptors. Following endocytosis, endosome acidification can be buffered by the positive PEI nitrogens and leads to swelling and rupture of the endosome, thus releasing the PEI-DNA complexes into the cytoplasm. Nuclear entry of these complexes is then facilitated by an unknown mechanism allowing the delivered plasmid to be transcribed. The resulting shRNAs are exported to the cytoplasm where they are processed into siRNAs capable of functioning in gene silencing.



In addition, modified ligand-conjugated PEIs have been designed to target specific cells, tissues or organs. Of particular interest in the context of filovirus infection, would be a galactose-conjugated PEI that targets liver cells expressing galactose-specific membrane lectins, such as the asialoglycoprotein receptor (ASG-R), which has been shown to function as a liver-specific receptor for MARV (Becker et al., 1995). One further approach is the possibility of targeted delivery to macrophages and dendritic cells using mannose-conjugated PEIs. Since these are very important early target cells for filovirus infection (Bray & Geisbert, 2005), if growth in these cells can be limited at an early stage of infection then spread to secondary target tissues could also be suppressed. The possibility of such cell-type specific targeting in the context of therapeutic approaches could, therefore, enhance the efficacy of treatment by ensuring uptake by relevant cell types.

## 1.4 Objectives and Hypothesis

Filoviruses are widely known for their ability to cause a devastating haemorrhagic fever, as well as for their unusually high case fatality rates. However, this virus family is also a characterized by a dramatic range in virulence phenotypes with the most notable example being the different between REBOV and ZEBOV. To date there is no evidence of human disease caused by infection with REBOV, despite evidence that at least 8 people were infected during the various epizootics. This is in stark contrast to the closely related ZEBOV, which has case fatality rates up to 90%; however, there is currently no molecular basis for this difference.

The availability of reverse genetics technology presents a number of opportunities to carry out studies into the molecular basis for these differences in pathogenicity among

filoviruses. Previous work with minigenome systems for ZEBOV and *Lake Victoria marburgvirus* (MARV) has demonstrated that these systems are uniquely capable of dissecting molecular aspects of transcription and replication and have, in fact, provided much of our current knowledge about these processes. Further, with the recent development of infectious clone systems for ZEBOV (Neumann et al., 2002a, Volchkov et al., 2001), we now have tools to study mutant EBOV in tissue culture and animal models. A similar system for REBOV would be extremely beneficial for pathogenesis studies in future. Its availability would not only be valuable as part of a comparative approach looking at complementary changes in both the ZEBOV and REBOV systems, but it is likely that the use of a low virulence background into which pathogenic determinants can be added will allow more sensitive detection of factors that enhance virulence or infectivity.

It was, therefore, the goal of this study to develop reverse genetics tools for REBOV, in light of the hypothesis that such systems for a low virulence EBOV will provide us with more sensitive tools for the identification of pathogenic determinants. Further we expected that the availability of these systems would provide us with powerful tools to explore factors related to both basic virus biology and the molecular basis for filovirus virulence.

In the absence of sufficient molecular data to undertake these final goals, this project began with the acquisition of complete genome sequence data for REBOV. In addition, the absence of reagents for REBOV necessitated the development of polyclonal antisera against several of the RNP complex proteins. With these tools in place the next objective was to establish a REBOV minigenome system and obtain information

regarding its optimization that could be applied to the development of more complex reverse genetics systems (i.e. the iVLP and infectious clone systems). Finally, it was our intent to apply the REBOV minigenome systems as a tool to study virus transcription and replication, as well as for use as screening tools for antiviral approaches capable of targeting transcription and replication. In particular, we wished to establish whether the RNP complex plays a role in determining viral virulence, as well as to explore protein specific contributions of the RNP to the requirement for VP30 in the regulation of EBOV transcription. Finally, this work aimed to examine the possibility of applying minigenome systems to the identification of potential siRNA target sequences capable of controlling viral infection and developing these identified siRNAs as potential therapeutic approaches both *in vitro* and *in vivo*. Thus the major objectives for this project have been as follows:

**Preliminary Objectives:**

- Full-length genome sequencing for REBOV
- Generation of anti-RNP complex antibodies

**Minigenome System Development:**

- Eukaryotic expression of RNP complex proteins
- Construction of T7 and Pol I-driven minigenome plasmids
- Establishment of minigenome systems
- Optimization of minigenome systems

**Minigenome Application**

- Comparison of filovirus RNP complex activities
- Investigation into the role of VP30 in regulating minigenome transcription

- Screening of potential siRNA targets in minigenome systems
- Validation of siRNA function in tissue culture
- Testing of siRNAs in a mouse model of ZEBOV infection

## 1.5 Significance of the Study

To date, the number of infectious cDNA clone systems for viruses causing VHF remains very limited. However, it is clear from the existing systems that they represent powerful tools to investigate many aspects of the virus lifecycle as well as to study virus-host interactions. Since the pathogenic features of REBOV are so dramatically different from that of all other filoviruses, despite relatively limited differences in sequence, this virus presents an important starting point to investigate the molecular basis for the extreme virulence of the other filovirus species. Through this work reverse genetics tools for this important virus have been developed and using them we have begun to address fundamental questions regarding filovirus transcription and replication, identify factors that may contribute to the decreased virulence of REBOV and investigate the possible application of minigenome systems for antiviral screening. Having established the suitability of minigenome systems for this application in the REBOV system, we were able to then transfer this technology, through collaborations, to existing ZEBOV minigenome systems where we were able to identify siRNAs capable of controlling virus replication in cell culture and which appear to show promise when used as a treatment in a mouse model of ZEBOV infection.

Future investigations using these systems will no doubt be instrumental for studying further aspects of viral transcription and replication, as well as their regulation.

However, perhaps the greatest challenge will be in the development of the infectious clone system and its application to identifying molecular determinants of pathogenesis. Since filovirus pathogenesis is clearly a multifactorial process, it is possible that the removal of individual virulence factors may be masked by what, in the case of ZEBOV, is an already extremely virulent system. In this application we expect the REBOV systems to be of particular value, as it is likely that the use of a low virulence background will then provide a more sensitive detection system for the incorporation of virulence determinants. This is in addition to simply providing a confirmatory system for evaluation of any putative pathogenic determinants identified in the existing ZEBOV system. The reverse genetics tools created as part of this work, as well as the information gathered through their optimization now form the basis for our approach toward generating a REBOV infectious clone system. Finally, if the determinants of filovirus virulence and can be adequately identified, the possibility remains for defined mutants to be created, using the infectious clone technology, which would then have potential applications as vaccine candidates. Considering its close phylogenetic relationship to the pathogenic filoviruses, and in light of its dramatically reduced pathogenic potential, the REBOV infectious clone system would be a likely starting point for any such undertaking.

# 2.0 Materials and Methods

## 2.1 Cells

### 2.1.1 Eukaryotic Continuous Cell Lines

VeroE6 (ATCC CRL-1586) are African green monkey (*Cercopithecus aethiops*) kidney cells. They were maintained in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% (v/v) heat-inactivated (30 min, 50°C) fetal bovine serum (FBS), 2mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco).

293T human embryonic kidney carcinoma cells, which are a simian virus 40 large T antigen-containing derivative of the HEK293 cell line (DuBridge et al., 1987), were kindly provided by Dirk Lindemann (Technischen Universität Dresden). Cells were maintained in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 2mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

All cell lines were grown and maintained at 37°C and 5% $CO_2$ in a humidified (95%) incubator.

### 2.1.2 Bacterial Cells

#### 2.1.2.1 Bacterial Genotypes

*Eschericia coli* (*E. coli*) of the XL-1 Blue strain (genotype: *recA1 endA1 gyr96 thi-1 hsdR17 supE44 relA1 lac* [*F'proAB lacI$^q$ZΔM15 Tn10(Tet$^r$)*]) were used for all routine cloning procedures. These cells are endonuclease (*endA*) deficient, which improves the quality of DNA obtained by eliminating non-specific digestion by Endonuclease I, as well as recombination (*recA*) deficient, which improves insert

stability. The *hsdR* mutation further prevents cleavage of cloned DNA by the *Eco*K endonuclease system, while the *lacI*q*ZΔM15* gene on the F′ episome allows for blue-white selection.

BL-21 *E. coli* [genotype: *F- ompT hsdS*(*r-B m-B*) *gal dcm*] were used for the production of recombinant REBOV proteins for antibody production. Following expression plasmid isolation from XL-1 Blue *E. coli*, all vectors were re-transformed into chemically competent BL-21 prior to the expression of the recombinant proteins. BL21 cells are deficient in the OmpT (extracellular) and Lon (intracellular) proteases and, therefore, their use minimizes recombinant protein degradation during both expression and purification.

GT-116 [genotype: recA1 endA1 $F^-$ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 Δdcm ΔsbcC-sbcD] is an SbcCD complex deletion strain engineered to support the growth of plasmid DNAs carrying hairpin structures, which are known to be unstable in *E. coli* due to recognition and cleavage by SbcCD. As such, these strains were used for all cloning involving the construction of psiRNA vectors for the expression of small hairpin RNAs (shRNAs).

### 2.1.2.2 Transformation Competence

With the exception of Top10, all the above listed bacterial strains were made competent chemically by the addition of transformation and storage solution (TSS) buffer (Chung et al., 1989). For this procedure, cells were grown overnight at 37°C in Lauria-Lenox broth with 0.5% (w/v) NaCl (LB) and diluted 1:100 into fresh LB. These cells were then grown at 37°C with shaking (~200 rpm) to an optical density at 600 nm

($OD_{600}$) of 0.5-0.8. Calls were then incubated on ice for 30 min prior to centrifugation at 3000 x *g* at 4°C for 10 minutes. The culture supernatant was discarded and the cell pellet was resuspended in a 1:10 volume of TSS buffer. The TSS buffer was prepared by combining 85% (v/v) LB broth, 10% (v/v) polyethylene glycol (PEG) 8000, 5% (v/v) dimethyl sulfoxide (DMSO) and 50 mM $MgCl_2$, with filter-sterilization prior to use. Cells were then frozen on dry ice in 100 µL aliquots and transferred to -80°C for storage.

### 2.1.3 Viruses

*Reston ebolavirus* (REBOV) (strain Pennsylvania) and *Zaire ebolavirus* (ZEBOV) (strain Mayinga) were kindly provided by the Special Pathogens Branch of the Centers for Disease Control and Prevention (Atlanta, Georgia). All experiments using infectious viruses were performed in the BSL-4 laboratory at the National Microbiology Laboratory, Health Canada.

## 2.2 Standard Molecular Techniques

### 2.2.1 RNA Extraction

For RNA isolation, VeroE6 cells were infected in the high containment laboratory at the Canadian Science Centre for Human and Animal Health with REBOV at a multiplicity of infection (MOI) of 0.01 pfu. Following an adsorption period of 1 h, DMEM (Invitrogen, Burlington, Ontario) containing 2% (v/v) FCS (Invitrogen, Burlington, Ontario) was added and the cells were incubated for 14 days. Virus was pelleted by centrifugation at 52,000 x*g* in an SW41 rotor for 30 min. The virus pellet was lysed and inactivated in a guanidinium isothyocyanate-based buffer (RTL buffer,

RNeasy kit, Qiagen, Mississauga, Ontario) and taken out of containment. Subsequently, the RNA was extracted using the RNeasy kit (Qiagen, Mississauga, Ontario) according to the manufacturer's instructions. Finally, RNA was eluted in RNase-free water and stored in 10 µl aliqots at -80°C.

### 2.2.2 Primers

Key primers sequences used for open reading frame cloning, minigenome and full-length genome plasmid construction and protein expression constructs are listed in Appendix A. Primers sequences are based on GenBank sequence data [ZEBOV: AF272001, MARV: Z12132, REBOV AF522874] and were synthesized by the DNA Core Facility at the Canadian Science Centre for Human and Animal Health or by Operon Biotechnologies Inc.

### 2.2.3 Reverse Transcription

First strand synthesis of cDNA was accomplished for all experiments using the SuperScript II Reverse Transcriptase system (Invitrogen) according to the manufacture's instructions. SuperScript II is an engineered version of the M-MLV RT with reduced RNase H activity capable of synthesizing complementary DNA strands from single-stranded RNA, DNA, or RNA:DNA hybrids. This modification eliminates the degradation of RNA molecules during cDNA synthesis, thus resulting in increased cDNA yields and more full-length cDNA synthesis. However, subsequent RNaseH digest is required to remove the RNA prior to use for PCR applications.

Reactions mixtures were set up by adding the following reagents to a nuclease-

free microcentrifuge tube:

1µl Gene-specific forward primer (200ng)

1µl Viral REBOV RNA (35 ng; prepared as described above)

1µl dNTP Mix (10 mM each)

This mixture was heated to 65°C for 5 min and then quickly chilled on ice in order to denature the template RNA. The reaction was then collected using a brief centrifugation and the following components added:

4 µl 5X First-Strand Buffer

2 µl 0.1M DTT

1 µl RNase Inhibitor (40 units/µl)

The contents were mix gently and incubated at 42°C for 2 min to heat the buffer prior to polymerase addition. Once the buffer was at 42°C, 1 µl (200 units) of SuperScript II RT was added and the reaction mixed by pipetting. This final mixture was incubated at 42°C for 50 min to allow cDNA synthesis to occur. Following DNA synthesis the reaction was heat inactivated by incubation at 70°C for 15 min, after which 1 µl (2 units) of *E. coli* RNase H was added. The reaction mixture was incubated for a further 20 min at 37°C to allow for digestion of the RNA in RNA:DNA hybrids prior to use in PCR.

### 2.2.4 Polymerase Chain Reaction

For subsequent amplification by polymerase chain reaction (PCR) 10-20% of the RT first-strand synthesis reaction for PCR was used. For this process, Pwo DNA

polymerase was used (Roche). This polymerase was originally isolated from the hyperthermophilic archaebacterium *Pyrococcus woesei* and is a highly prossessive 5'–3' DNA polymerase with a 3'–5' exonuclease (proofreading) activity.

As a result of this proofreading Pwo demonstrates an approximately 18-fold increase in fidelity over Taq. However, it also results in degradation of template and primer DNA in the absence of dNTPs. Therefore, these reactions are always set up as two mixtures, one containing DNA, primers and nucleotides and the other containing the buffer and enzyme. These mixtures are not combined until just before cycling to avoid these degradation events.

The individual reaction mixtures were set up on ice in sterile microcentrifuge tubes using the components listed in Table 6.

**Table 6. Standard composition of a Pwo PCR reaction**

| | **Volume** | **Component** |
|---|---|---|
| **Mixture #1** | 1-2 µl<br>1 µl<br>1 µl<br>2 µl<br>x µl | First-strand cDNA<br>Forward primer (200 ng/µl)<br>Reverse Primer (200 ng/µl)<br>dNTPs (10 mM each)<br>Sterile distilled water |
| | 50µl | Total |
| **Mixture #2** | 10 µl<br>0.5 µl<br>x µl | 10X Reaction Buffer with 20 mM MgSO4<br>Pwo Enzyme (2.5 U/µl)<br>Sterile distilled water |
| | 50µl | Total |

After setup, Mix 1 and Mix 2 were combined in a 0.2 mL thin-walled PCR tube. Samples were then mixed and centrifuged briefly to collect the sample at the bottom of the tube and immediately thermocycled.

Melting temperature values for a given primer pair was determined using the

following formula (Rychlik & Rhoads, 1989):

$$T_m = 2(\#\ of\ A\ residues + \#\ of\ T\ residues) + 4(\#\ of\ G\ residues + \#\ of\ C\ residues)$$

Alternatively, if the primers contained mismatched base pairs, such as for site-directed mutagenesis, the following formula (Stratagene; Quick Change mutagenesis Handbook) was applied:

$$T_m = 81.5 + 0.41\ (\%\ GC\ content) - (675 / \#\ primer\ length) - \%\ mismatch$$

Based on these calculations the reaction was cycled in either a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer) or a Biometra T1 (Mason Technology) thermocycler according to the conditions shown in Table 7. However, in no case was the annealing temperature used allowed to exceed 72°C regardless of the calculated value. Following cycling, 10% of the PCR reaction was typically analyzed for successful amplification as described below.

**Table 7. Standard cycling conditions for PCR amplification using Pwo**

| Step | Cycles | Temperature | Time |
|---|---|---|---|
| Initial Denaturation | 1x | 94°C | 2 min |
| Denaturation | 40x | 94°C | 30 s |
| Annealing | | 45 – 72°C* | 30 s |
| Elongation | | 72°C | 1min/kb |
| Final Elongation | 1x | 72°C | 7 min |

***Annealing temperature was selected to be 5°C less the predicted melting temperatures of the primers used.**

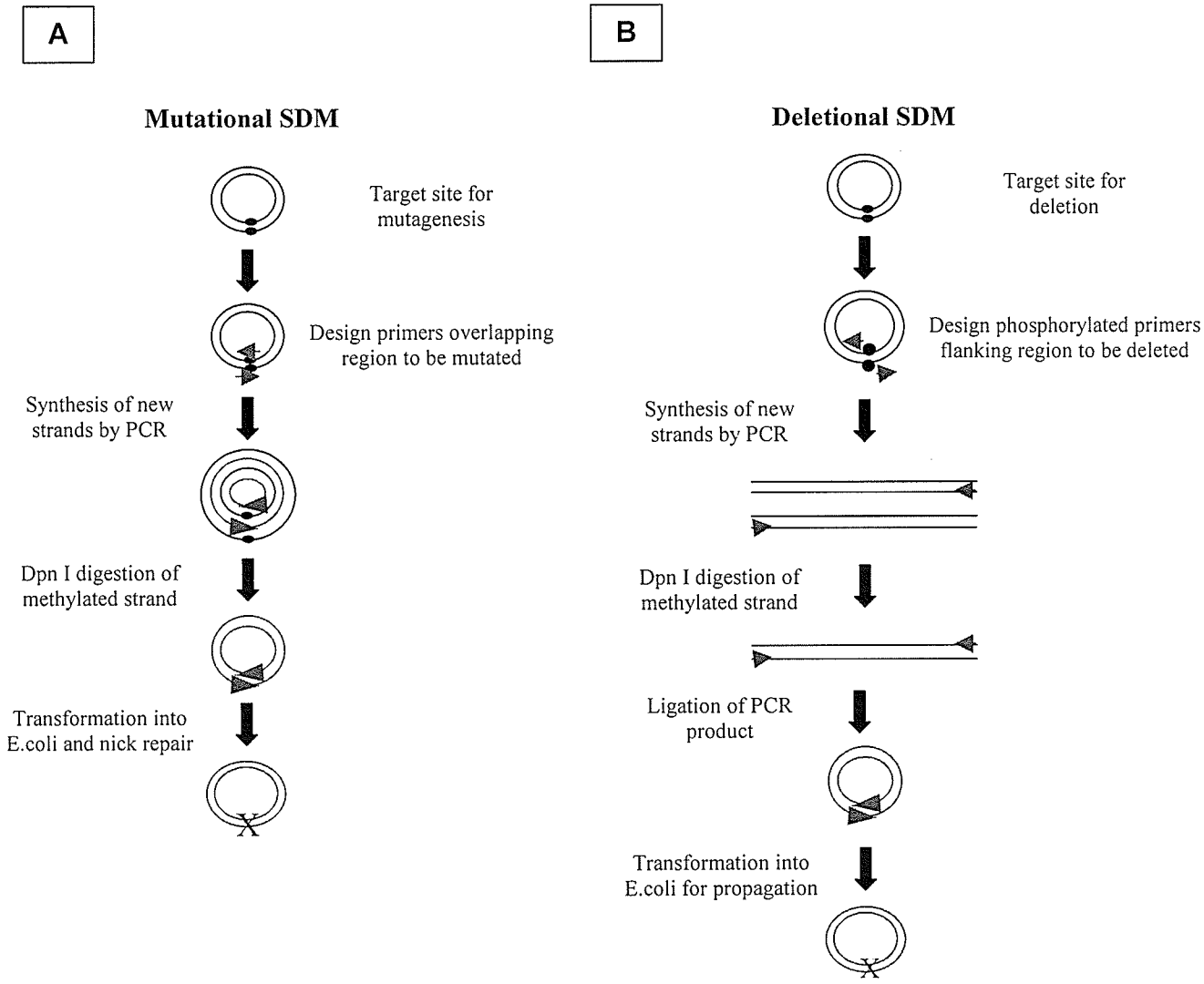
### 2.2.5 Site-Directed Mutagenesis

Site-directed mutagenesis (SDM) used in these studies was either deletional or mutational in nature, with mutational site directed mutagenesis used to modify or remove single bases (Figure 12A) and deletional used to remove larger sections of undesirable sequence (Figure 12B). Both forms of SDM were performed using the Quickchange kit (Stratagene) and for mutational SDM the manufacturer's recommendations for primer design and the manufacturer's protocol were followed. For deletional SDM, primers of approximately 20-25 bp were designed according to conventional guidelines for primer design. These primers flanked the region to be deleted and defined this area based on their 5' end sequences (Figure 12B). The 5' ends of these primers were phosphorylated such that following amplification of the target using Pfu turbo (according to the directions in the Stratagene manual) these ends could be joined using T4 DNA ligase (Invitrogen). Prior to ligation the reaction was digested with Dpn I according the Quickchange protocol and separated by agarose gel electrophoresis. Depending on the presence of contaminating amplicons the correctly sized amplicon was either gel extracted, as described below, or used directly for ligation. Ligation was performed using T4 DNA ligase (Invitrogen) by incubation at 14°C overnight. The ligated material was then transformed into competent Xl-1 Blue *E.coli* and the mutagenized plasmid recovered as described below.

### 2.2.6 Amplicon Analysis

Amplicons generated by PCR were visualized using TAE agarose gel electrophoresis. For analytical separation of DNA fragments >500bp, 1% (w/v) agarose

**Figure 12. Strategies for site-directed mutagenesis.** For mutational site-directed mutagenesis primers were designed overlapping the site to be mutated. Through PCR amplification new strands containing the primer-encoded changes were synthesized and the template strands subsequently removed by DpnI digestion. Transformation of the PCR products into *E. coli* then leads to nick repair and the generation of the new, altered plasmid. Alternatively, if regions of plasmid are to be removed deletional mutagenesis can be used. In this approach primers are designed flanking the region to be removed, such that PCR amplification leads to the production of linear products lacking the region to be deleted. DpnI digestion of the template DNA and ligation of the PCR products leads to formation of the new plasmid, which is transformed into *E. coli* for propagation.




gels were prepared, while for fragments <500bp, 2% (w/v) agarose gels were used. Gels were prepared by dissolving the appropriate amount of agarose in 1x TAE [Appendix B] and heating until completely dissolved. Ethidium bromide (EtBr) solution was added to a

final concentration of 0.66 μg/mL once the gel was cooled and poured into an appropriately sized form. For preparative extractions of DNA fragments >500bp, 0.8% (w/v) agarose gels were prepared, while for fragments <500bp, 1% (w/v) agarose gels were used. Gels were prepared as above, except that EtBr was not added directly to the gel. Prior to loading samples were mixed with 6x Nucleic acid gel loading buffer [Appendix B] to a final 1x concentration.

After running these gels were immersed in a 1 μg/mL solution of EtBr for 20-30 min until bands were visible. Gels were run at 100-120V depending on the tank size, until bands were well separated, as indicated by the position of bromphenol blue (corresponds to ~200-400 bp) in the gel-loading buffer. Once fully separated DNA bands were visualized for analytical purposes using the short wavelength setting of a MacroVue UV-25 Hoefer transilluminator. In cases where the visualized fragment was excised for further use, visualization was achieved with a Mineralight Lamp Multiband handheld UV illuminator using the long wavelength setting in order to prevent damage to the DNA as a result of irradiation. The sizes of DNA fragments were determined by comparison with those of known size in the 1kb plus ladder (Invitrogen). Results were documented using a Fisher brand Polaroid Photo documentation camera.

### 2.2.7 Amplicon Clean-up

Following the generation of PCR products, as described above, amplicons were purified away from remaining nucleotides, primers and/or primer dimmers using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's directions. Alternatively, if significant levels of contaminating amplicons were present the band of

interest was excised and recovered using the QIAquick Gel Extraction kit (Qiagen) also according to the manufacturer's instructions.

### 2.2.8 Transformation by Heat-shock

In order to introduce plasmid DNA into competent *E.coli* a standard heat shock method was used. Prior to transformation vials of previously frozen chemically competent cells, which were either purchased or prepared as described above, were thawed on ice. To each tube 10μl of ligation reaction was added. These samples were gently mixed by stirring with a pipette tip and allowed to incubate on ice for 20-30 min. Subsequently, the cells were rapidly transferred to a 42°C heating block or water bath for 45–60 sec. The tubes were then immediately placed on ice and allowed to cool for 3 min after which 250 μl of SOC medium was added to each tube. Samples were then incubated for 45 min at 37°C without shaking, to allow expression of the plasmid-encoded antibiotic resistance gene, and finally plated on LB agar plate containing either 100 μg/mL ampicillin or 15 μg/mL kanamycin. Plates were incubated overnight at 37°C to allow adequate colony formation.

### 2.2.9 Plasmid Propagation

For small-scale generation of plasmids single bacterial colonies were picked and resuspended in 3-5 mL LB containing either 100 μg/mL ampicillin or 25 μg/mL kanamycin, depending on the plasmid resistance genes, and shaken overnight at 37°C and 250 rpm. Plasmid was then isolated from the culture using the QIAspin miniprep kit according to the manufacturer's instructions.

All plasmids propagated for large-scale recovery were started from a 1:1000 dilution of an overnight culture into 100 mL culture volume of LB containing either 100 μg/mL ampicillin or 25 μg/mL kanamycin, depending on the plasmid resistance genes. These cultures were then grown overnight at 37°C with 250 rpm shaking. Plasmids were subsequently isolated from the culture using the QIAfilter maxiprep kit according to the manufacturer's instructions. Additional buffer P1, P2 and P3 were prepared as required [Appendix B]. Following precipitation in 70% (v/v) ethanol the DNA pellets were resuspended in sterile distilled water for use.

### 2.2.10 Nucleic Acid Quantification

The concentrations of all DNA and RNA preparations were determined using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies). Based on the absorption spectra and $A_{260/280}$ values generated the purity of nucleic acid preparations was also determined.

### 2.2.11 SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gels were prepared and run using the Protean III Gel system (BioRad). Resolving and stacking gels were prepared by addition of the reagent in order as indicated in Table 8.

Ingredients were mixed gently and poured into glass plates. The resolving gel was overlaid with isopropanol in order to ensure a flat surface and to exclude air. After the gel was set (~15 min) the isopropanol was removed and a stacking gel overlaid. Once fully set gels were immersed in a Protean III Gel running tank containing 1x SDS-PAGE

**Table 8. SDS PAGE resolving and stacking gel composition**

| Component | 10% (w/w) Resolving Gel | 4% (w/w) Stacking Gel |
|---|---|---|
| Sterile distilled water | 4.4 mL | 3.2 mL |
| 40% Acrylamide (w/w)* | 3.0 mL | 500µl |
| 1.5M Tris-HCl (pH 8.8) | 2.5 mL | ----- |
| 0.5M Tris-HCl (pH 6.8) | ----- | 1.25 mL |
| SDS | 100 µl | 50 µl |
| 10% (w/v) APS | 50 µl | 25 µl |
| TEMED | 10 µl | 5 µl |
| Total | 10 mL | 5 mL |

*** 37.5:1 (w/w) ratio of acrylamide to N,N'-methylene bis-acrylamide**

running buffer [Appendix B] and run at 100V until adequate resolution in the desired size range was achieved, as indicated by the position of protein standards in the SeeBlue protein marker (Invitrogen).

To protein samples, including markers, 4x SDS-PAGE Gel-loading buffer with β–mercaptoethanol (BME) [Appendix B] was added to a final 1x concentration. Samples were then placed in an Eppendorf Thermomixer heating block at 100°C for 5 min and allowed to cool to room temperature before loading in order to ensure complete denaturation.

### 2.2.12 Electrophoretic Protein Transfer

Polyvinylidene difluoride (PVDF) was prepared by soaking in methanol for 5 min and then briefly wetted in Tris-glycine transfer buffer [Appendix B]. During this time absorbent mesh pads and filter papers were soaked in Tris-glycine transfer buffer [Appendix B] until thoroughly wetted. At this point the components were assembled such that the mesh pad was placed on clear ("cathode") face of a Protean III clamping frame (Biorad). Onto this was placed a wetted filter pad and the prepared PVDF. The

gel from which protein was to be transferred was then overlaid and rolled flat to remove any air bubbles. Another final filter pad and a final mesh pad were added to the top of the stack and the entire assembly was again rolled flat to remove any air bubbles before the clamping frame was closed and locked. The clamping frames were placed into the transblot module (Biorad) along with an ice block to prevent overheating of the unit. This entire assembly was placed in a plastic tank and the remaining space filled with Tris-glycine transfer buffer [Appendix B]. Electrophoretic transfer was performed overnight at 30V and 40 mA with constant stirring from a magnetic stir plate.

### 2.2.13 Western Blotting

Once protein transfer was complete PVDF membranes were blocked for 1 h at room temperature in 10% (w/v) skim milk in PBS with 0.01% (v/v) Tween 20 or overnight at 4°C in 10% (w/v) skim milk in PBS without Tween. The blot was rinsed once prior to use in PBS-Tween and then washed in PBS-Tween while primary antibody dilutions were prepared in 1% (w/v) skim milk in PBS-Tween. Following an incubation of 1 h at room temperature, the blots were washed three times for 10min in PBS-Tween. Secondary antibody dilutions were then prepared in 1% (w/v) skim milk in PBS-Tween and added to the blots. Following a further 1 h incubation at RT, blots were washed twice with PBS-Tween and 4x with PBS. Blots were then developed using the ECL Plus Detection System according to the manufacturer's directions (Amersham Biosciences). Hyperfilm ECL (Amersham Biosciences) was exposed to blots for between 10 sec and 15 min depending on signal strength before being automatically developed using a Feline 14 X-ray film processor (Fisher Industries Inc.).

### 2.2.14 Poly-D Lysine Coating

Due to the lack of adherence of many 293-derived cell lines, cell culture vessels for transfection of these cells were treated with Poly-D Lysine (Sigma). This reagent functions by increasing the number of positively charged sites on the plastic surface that are available for cell binding and thus improve cell adherence and minimize cell loss during manipulation. A 0.1 mg/mL solution of Poly-D Lysine (Sigma) was prepared in PBS. For 6 well plates 1mL of this solution was added to each well, while for 10 $cm^2$ dishes a minimum of 2 mL was added to each plate. Dishes were then incubated at 37°C for 30-60 min at 37°C. Subsequently, the wells were washed twice with $sdH_20$ prior to use. Used Poly-D lysine was collected, stored and reused up to 5 times before being discarded.

### 2.2.15 TransIT-LT1 Transfection

For transfection 2µl of TransIT-LT1 (Mirus) per µg of DNA to be transfected was added dropwise into 100ul OptiMEM (Invitrogen). This mixture was mixed thoroughly by vortexing and allowed to incubate for 15 min at RT. Subsequently DNA for transfection was added to the diluted TransIT-LT1 mixture. Tubes were mixed gently and allowed to incubate for a further 15 min at RT. Cells were washed once with OptiMEM serum-free medium and placed into fresh OptiMEM. The TransIT/DNA mixture was then added dropwise to cells and plates gently rocked to distribute the complexes evenly.

### 2.2.16 Electroporation of Mammalian Cells

VeroE6 cells were split 1:2 on the day prior to transfection into the required number of T75 flasks (Costar). The next day one T75 was used for each plasmid to be transfected. For each electroportation 10 μg of the DNA to be transfected was reduced to a volume of <20 μl using a DNA120 Speedvac (Thermo Electron Corporation). The DNA was then resuspended in 400 μl of ViaSpan Organ Preservation medium (Barr Laboratories Inc.). VeroE6 cells were washed twice in 10 mL of PBS and incubated with 2 mL Trypsin-EDTA (Gibco) until they began to detach. Remaining cells were washed from the plastic using 5 mL DMEM with 10% (v/v) FCS and centrifuged for 5 min at 100 x *g* at room temperature in a 50 mL conical tube. The resulting cell pellet was resuspended in the DNA/ViaSpan mixture by gentle shaking and placed into a 0.4 cm, gap cuvette. Cells were electroporated in a Genepulser Xcell (Biorad) unit with exponential decay setting and the following conditions: U = 230V, C = 950 μF, Ω = ∞. Following electroporation the cells were transferred into 6 mL DMEM with 10% (v/v) FCS and seeded into 6 well plates using 1mL per well or seeded directly into a T25 flask (Costar).

## 2.3 Full-length Genome Sequencing

### 2.3.1 Amplification of NP-GP and GP-L Regions

cDNA synthesis was performed using the first strand synthesis methodology described above, using previously described filovirus diagnostic primer sequences targeting the nucleoprotein, glycoprotein and RNA-dependent RNA polymerase genes (Sanchez & Feldmann, 1996, Sanchez et al., 1996) [Appendix A]. Two clones were

generated which covered larger regions between the NP and GP genes and the GP and L genes using these primers. All additional primers were based on sequence data generated from these experiments as the data were obtained and were used to generate smaller clones covering these and other remaining regions of the genome. For REBOV these data are now listed in GenBank under Accession #AF522874.

All products were cloned into the pCR3.1 TA cloning vector (Invitrogen, Burlington, Ontario) for sequence analysis. At least 3 clones for each region were analyzed. Subsequently, these sequence data were confirmed by direct sequencing of independently synthesized overlapping PCR amplicons, which covered the entire genome at least twice, with the exception of the 3' and 5' ends.

### 2.3.2 Genome End Amplification

To determine the genomic ends, viral genomic RNA molecules were circularized by ligating the 3' and 5' ends of the genome using T4 RNA ligase (Roche). RT and PCR reactions were performed using 5' trailer-specific upstream (genome position 18497-18520) and 3' leader-specific downstream primers (genome position 147-129). These ~540 nucleotide PCR products were cloned into the pCR3.1 TA vector for sequence determination. In total, 30 clones were sequenced to determine the exact 3' and 5' ends of the genome.

### 2.3.3 Sequencing of Amplicons and Constructs

Sequencing was performed by the DNA Core facility of the Canadian Science Centre for Human and Animal Health. An automated ABI 377 sequencer (Applied

Biosystems, Foster City, California) was used with materials for sequencing submitted in the amounts and concentrations listed in Table 9.

**Table 9. Sample requirements for sequencing**

| | **Concentration** | **Volume/reaction** |
|---|---|---|
| Plasmid Template | 150 μg/μl | 5 μl |
| PCR Products | 50 μg/μl | 5 μl |
| Primer | 1 μM | 5 μl |

### 2.3.4 Sequence Analysis

Database searches and sequence comparisons were performed using the National Center for Biotechnology Information BLAST search programs. GenBank accession numbers used in the analysis of sequence data were for the complete genome sequences of ZEBOV, strain Mayinga (AF272001), MARV, strain Musoke (Z12132) and REBOV Reston, Philippines (AB050936). The Chromas sequence analysis program (Griffith University; Version 1.41) was used to view ABI sequence files and the LASERGENE sequence analysis package (DNASTAR Inc.; Version 4.01) was used for the analysis and alignment of sequence data.

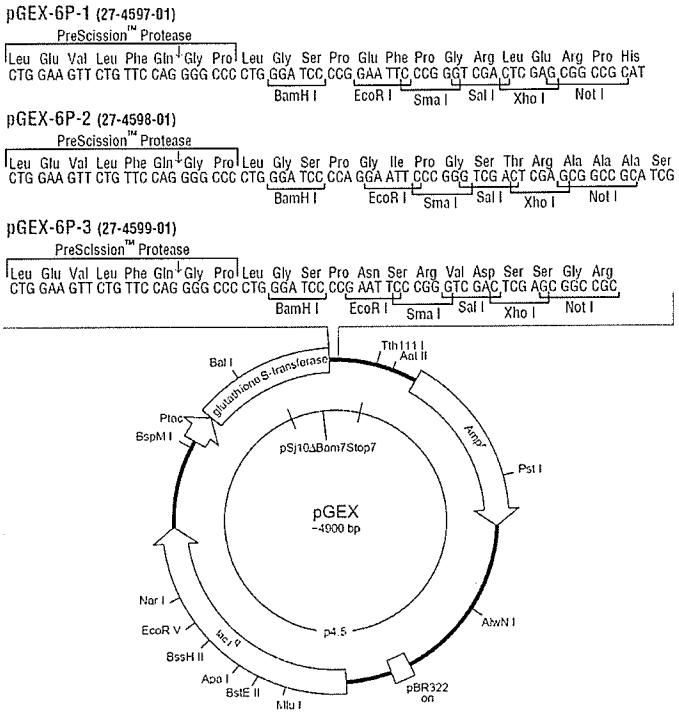
## 2.4 Antibodies

### 2.4.1 Production of anti-REBOV Antibodies

#### 2.4.1.1 Cloning of Protein Open Reading Frames and Peptide Fragment Sequences

The open reading frames for VP35 and VP30, as well as peptide fragments from NP and L, were expressed for use as antigen using the pGEX-6P series of vectors (Figure 13) (Amersham). Peptides were selected based on analysis of the NP and L amino acid sequences using the Kyte-Doolittle method (Kyte & Doolittle, 1982). For NP the peptides clones covered the regions corresponding to amino acids 80-149 (NP1) and 490-674 (NP2) and for L the peptides clones corresponded to amino acids 1-56 (L1) and 1643-1758 (L2). The sequences of these peptides are listed in Table 10. Each ORF or peptide fragment was cloned into the multiple cloning site (MCS) in frame with the upstream glutathione–S-transferase (GST) gene. For VP35 *Eco*RI was used while for VP 30 *Eco*RI and *Xho*I were used. All NP and L peptide fragments were cloned into the *Eco*RI restriction site.

**Figure 13. pGEX-6P series of GST fusion protein expression vectors.** *Reston ebolavirus* protein open reading frames and peptide fragments were cloned in frame with the GST protein, encoded in the pGEX vectors, using the vector version containing the desired restriction sites in the correct context.

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### 2.4.1.2 Expression of GST Fusion Proteins

Overnight cultures of BL-21 bacteria containing each of the fusion protein plasmids were diluted 1:100 into 1L of fresh LB with 100 μg/mL ampicillin and grown at 30°C with shaking at 250 rpm until they reached an $OD_{600}$ of 0.6. At this time Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 100mM to induce protein expression and the cultures were incubated for a further 4 hrs at 30°C. Following this the cultures were centrifuged at 7700 x *g* for 10 min and the supernatants discarded. The resulting cell pellets were frozen overnight at -20°C prior to lysis and purification.

**Table 10. Peptides used for antibody development**

| Peptide Name | Amino acid Position | Length (aa) | Estimated Mass (kD) | Peptide Sequence |
|---|---|---|---|---|
| NP #1 | 80-149 | 70 | 7.87 | HHAYQGDYKLFLESNAVQYLEGHGFKFE LRKKDGVNRLEELLPAATSGKNIRRTLAA LPEEETTEANAGQ |
| NP #2 | 490-674 | 185 | 20.96 | DHEDDNKAFEPQDSSPQSQREIERERLIHPP PGNNKDDNRASDNNQQSADSEEQGGQYN WHRGPERTTANRRLSPVHEEDTLMDQGD DDPSSLPPLESDDDDASSSQQDPDYTAVAP PAPVYRSAEAHEPPHKSSNEPAETSQLNED PDIGQSKSMQKLEETYHHLLRTQGPFEAIN YYHMMKDE |
| L #1 | 1-56 | 56 | 6.48 | MATQHTQYPDARLSSPIVLDQCDLVTRAC GLYSSYSLNPQLRQCKLPKHIYRLKFD |
| L #2 | 1643-1758 | 116 | 13.63 | WRSRKSRKTQDHNDFSRGDGTLTEPVRKF SSNHQSDEKYYNVTCGKSPKPQERKDFSQ YRLSNNGQTMSNHRKKGKFHKWNPCKM LMESQRGTVLTEGDYFQNNTPPTDDVSSP HR |

### 2.4.1.3 Purification of GST Fusion Proteins

Frozen bacterial pellets were first resuspended in 50ul of PBS per mL of culture volume. To this sample 0.1 volumes of 10 mg/mL chicken egg white lysozyme (Sigma)

was added and the sample was vortexed briefly. Samples were then incubated at room temperature for 5 min and sonicated on ice for 3-5 min until clearing of the suspension was observed. At this time 20% (v/v) Triton X-100 was added to the culture to give a final concentration of 1% (v/v) Triton X-100. The lysates were briefly mixed and centrifuged at 15,000 x *g* for 15 min to clear insoluble material. The supernatants from these lysates were moved to a fresh tube and 2 mL of a 50% (v/v) sepharose slurry (Amersham) added. This was incubated at 4°C with gentle rocking for 30 min and then the tube was centrifuged at 500 x *g* for 5 minutes. The supernatant was removed and the sepharose beads were washed 3 times with 10 bed volumes of PBS. Then 1 mL of GST elution buffer (Amersham) was added and the sample transferred to 1.5 mL microfuge tubes. The sample was incubated at room temperature for 10 min with occasional mixing. After this the sample was centrifuged at 500 x *g* for 5 min and the buffer removed. This elution process was repeated twice with fresh aliquots of elution buffer. A final 1 mL of elution buffer was added and the beads incubated overnight at 4°C with an additional 30 min incubation at room temperature the following day. The sample was centrifuged at 500 x *g* for 5 min and the elution buffer removed. The used sepharose beads were stored at 4°C in a minimal amount of elution buffer.

In order to determine which samples contained the fusion proteins of interest and during which steps protein may have been lost, samples from each step of the purification process were analyzed by SDS-PAGE through a 10% (w/w) acrylamide gel. Following separation, the gels were stained in a 0.25% (w/v) solution of Coomassie brilliant blue R-250 (Sigma) stain [Appendix B] for > 1 h and subsequently destained in Coomassie destain [Appendix B] until a sufficiently clear background was obtained (~4 h).

#### 2.4.1.4 Immunization of BALB/c Mice with Fusion Proteins

The elution fraction containing the fewest contaminants was selected for each protein and diluted to a concentration of 0.1 μg/μl in PBS. Prior to use complete Freund's adjuvant (CFA) was vortexed for 2 min at high speed to disperse the *Mycobacterium tuberculosis* bacilli. From these protein stocks 500μl (50 μg) was emulsified together with 0.5 mL of CFA by passing it between a pair of glass syringes connected by a Luer-Lok double-ended connector until white in colour and homogenous. Three female BALB/c mice per antigen were injected with 0.1 mL of emulsion at three intramuscular (i.m.) sites on the hind limbs for a total of 0.3 mL (~15 μg) per animal. A booster immunization was prepared as above using incomplete Freund's adjuvant. This was administered 4 weeks following the initial immunization as a single 0.3 mL injection subcutaneously at the back of the neck. Fourteen days later, immunized mice were bled by cardiac puncture and the serum separated, by centrifugation at 2,000 x *g* for 15 min at room temperature, following clotting. Serum from each group was pooled, moved to clean microcentrifuge tubes and aliquoted prior to storage at -20°C. These animal experiments were performed on animal use documents approved by the Canadian Science Centre for Human and Animal Health - Animal Care committee in accordance with the guidelines of the Canadian Council on Animal Care.

### 2.4.2 Additional Antibodies

A complete list of antibodies used in these studies, including evaluated methods, working dilutions, suppliers and sources animal, is detailed in Table 11.

**Table 11. Antibodies used for the detection of filoviruses**

| Antibody Target | Source Animal | Supplier | Specificity | Evaluated Methods | Working Dilution |
|---|---|---|---|---|---|
| **Primary Antibodies** | | | | | |
| α-Actin (clone AC-40) | Mouse | Sigma | Actin | Western Blot | 1:500 |
| α-ZEBOV NP | Mouse | Philipps Universität Marburg* | ZEBOV | Western Blot | 1:100 |
| α-ZEBOV VP40 | Rabbit | University of Tokyo¥ | ZEBOV | IFA | 1:200 |
| α-REBOV NP 2 | Mouse | | REBOV | Western Blot | 1:2000 |
| | | | | IFA | 1:400 |
| α-REBOV VP30 | Mouse | | REBOV | Western Blot | 1:4000 |
| | | | | IFA | 1:1000 |
| | | | ZEBOV | Western Blot | 1:100 |
| α-REBOV VP35 | Mouse | | REBOV | Western Blot | 1:4000 |
| | | | | IFA | 1:1000 |
| | | | ZEBOV | Western Blot | 1:10 |
| α-MARV VP30 | Guinea Pig | | MARV | IFA | 1:100 |
| α-MARV VP35 | Mouse | | MARV | IFA | 1:1000 |
| **Secondary Antibodies** | | | | | |
| α-Mouse IgG; HRP | Goat | KPL | Mouse IgG | Western Blot | 1:30,000 |
| α-Mouse IgG; HRP | Goat | Jackson Immunoresearch Laboratories | Mouse IgG | Western Blot | 1:50,000 |
| α-Mouse IgG; Alexa 488 | Goat | Molecular Probes | Mouse IgG | IFA | 1:200 |
| α-Guinea pig IgG; FITC | Rabbit | Sigma | Guinea pig IgG | IFA | 1:300 |
| α-Rabbit IgG; FITC | Goat | Sigma | Rabbit IgG | IFA | 1:250 |

* The kind gift of Dr. Stephan Becker (Philipps Universität Marburg)
¥ The kind gift of Dr. Ayoto Takada (University of Tokyo)

## 2.5 Minigenome Systems

### 2.5.1 REBOV Minigenome

#### 2.5.1.1 REBOV Minigenome Cloning

##### 2.5.1.1.1 Pol I Minigenome Construction

In order to generate a Pol I-driven REBOV minigenome construct, the viral 3' and 5' non-coding regions, referred to as the leader and trailer, respectively, were cloned flanking either a chloramphenicol acetyl transferase (CAT) or a green fluorescent protein (GFP) reporter gene. Both CAT and GFP open reading frames (ORFs) were PCR amplified from existing expression constructs kindly provided through collaboration with Dr. Ramon Flick (University of Texas Medical Branch). This cassette was produced by ligation of a *Bbs*I digested leader amplicon to CAT or GFP amplicons bearing compatible overhangs generated by *Bsa*I (CAT) or *BsmB*I (GFP) cleavage, respectively. The resulting fragment was PCR amplified and cloned into the human Pol I promoter and terminator-containing vector, pRF240 (Flick et al., 2003a, Flick et al., 2003b). This construct was cleaved with *Bbs*I and the trailer was inserted, again using overhangs generated by *Bbs*I cleavage. The resulting construct contained either a CAT or GFP minigenome cassette in vRNA orientation and consisted of the following elements: Pol I promoter - trailer - reporter - leader - Pol I terminator. In order to construct a complementary RNA (cRNA)-transcribing Pol I-driven minigenome, the entire reporter cassette (consisting of the CAT gene flanked by the leader and trailer regions) was amplified by PCR from the corresponding vRNA construct. This fragment was then inserted into pRF240 in a directional fashion using overhangs generated using *Bbs*I. The resulting construct contained the CAT minigenome cassette in cRNA orientation and

consisted of the following elements: Pol I promoter - leader - reporter - trailer - Pol I terminator.

#### 2.5.1.1.2 vRNA-Oriented T7 Minigenome Construction

Generation of the REBOV vRNA-oriented T7-driven minigenome construct was based on similar systems established for ZEBOV and MARV (Muhlberger et al., 1998, Muhlberger et al., 1999). This plasmid was produced by sub-cloning the reporter cassette from a vRNA-oriented Pol I-driven CAT construct into a T7 promoter-containing pBluescript-based vector (Stratagene), which also contained hepatitis delta virus (HDV) ribozyme and T7 terminator sequences. These elements were inserted into pBluescript using *Eag*I and *Sac*I following PCR amplification from a T7-driven vesicular stomatitis virus genome-containing plasmid (kindly provided by Dr. Jack Rose, Yale University). The HDV-T7 terminator fragment also contained an upstream *Bbs*I site, which produced overhangs compatible to the viral leader. Into this construct was then inserted the CAT reporter cassette (consisting of the CAT gene flanked by the leader and trailer regions) amplified from the vRNA-oriented Pol I minigenome using compatible ends produced by *Bbs*I and *Kpn*I cleavage. Subsequent site deletional mutagenesis was performed, as described above, to remove additional nucleotides, including the *Kpn*I site, between the T7 promoter and the viral trailer. Based on the primer design [Appendix A], constructs were generated in which either 0, 1, 2, or 3 G residues were left downstream of the T7 promoter in order to assess the impact of these approaches on minigenome activity. The final construct consisted of the following elements: T7 promoter – (0-3 G) - trailer - reporter - leader - HDV ribozyme - T7 terminator.

### 2.5.1.2 Helper Plasmid Constructs

#### 2.5.1.2.1 Cloning

The open reading frames for the NP, VP35, VP30 and L genes of REBOV were generated using the RT and PCR methodologies described above using sequence specific primers and cloned into the vector pCAGGS. This vector mediates a particularly high level of foreign gene expression by placing it under the control of a strong composite chicken β-actin derived promoter and including an SV40 origin of replication, which allows vector replication in the nuclei of T-antigen containing eukaryotic cells (i.e 293T) (Niwa *et al.* 1991). The entire ORF for NP and VP35 were cloned using *Eco*RI, while the VP30 ORF was cloned using *Eco*RI and *Xho*I. In the case of the L gene, the construct was cloned in two steps using the vector *Eco*RI and *Nhe*I sites. Since digestion of the insert with *Eco*RI was not possible, due to the presence of multiple cleavage sites, an *Eco*RI compatible overhang was generated using *Bsa*I. An *Nhe*I 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 using this *Nhe*I site as well as the vector *Bgl*II site. All constructs were sequenced in order to confirm that the ORFs were intact and that no mutations had been introduced.

#### 2.5.1.2.2 Detection by Immunofluorescence Assay

To determine whether the cloned helper plasmids were expressed correctly, and whether the antisera generated against the REBOV RNP complex proteins would detect their targets, the anti-REBOV antisera described above were tested by immunofluorescence assay (IFA). Eight chamber LABTEK slides (Nunc) were coated

with by the addition of 250ul/well of 0.1 mg/mL Poly-D-Lysine (Sigma) prepared in PBS. Following an incubation of 30-60 min at 37°C, wells were washed twice with $sdH_20$. To each well 300µl of DMEM with 10% (v/v) FCS was added along with 200µl of 293T cells prepared by suspension of an 80% confluent T75 flask (Costar) into 6 mL. These cells were allowed to incubate overnight at 37°C prior to transfection with 0.5 µg of either pCAGGS-NP, pCAGGS, VP35 or pCAGGS-VP30 using TransIT-LT1 (Mirus) ,as described above. Following transfection, these samples were incubated for 72 h prior to IFA. Samples were then fixed for 15min in 2% (w/v) paraformaldehyde (PFA) and washed three times with PBS for 5 min. In order to facilitate intracellular staining, the cells were permeabilized in PBS containing 0.1% (v/v) Triton X-100 for 10min. Cells were washed a further three times with PBS for 5 min prior to incubation with primary antibody diluted in PBS. For each well 120 µl of antibody was prepared using 1:100 to 1:1000 dilutions of NP, VP35 or VP30 antisera. These samples were incubated overnight at 4°C and washed three times with PBS for 5 min prior to incubation with the secondary antibody. In all cases 120 µl/well of 1:200 goat-α-mouse conjugated to Alexa 488 (Molecular Probes) diluted in PBS was used. Samples were incubated for 1hr at RT and then washed three times with PBS for 5 min. The antibodies were then fixed in 2% (w/v) PFA for 5 min and washed once with PBS for 10 min. Finally, the wells were filled completely with PBS for viewing by UV microscopy.

### 2.5.1.2.3 Detection by Western Blotting

In order to determine whether the REBOV antisera could detect the REBOV proteins against which they were raised in a denatured context, preparative western blots

were run and a variety of antibody dilutions tested. For this protocol 10% (w/w) acrylamide gels were cast containing a 2-well preparative comb. Approximately 25% of a 6 well dish worth of harvested lysate in a 100µl volume was loaded onto ½ of a gel and gels were run at 100V until sufficient separation in the range of 30-75 kD was achieved. Subsequently, the proteins were transferred to PVDF by wet electrophoretic transfer and prepared for Western Blot as described above. The sera raised against REBOV proteins were used as primary antibodies in dilutions from 1:100 to 1:4000. The secondary antibody was a goat-anti-mouse IgG conjugated to HRP (KPL) used in either a 1:10,000 dilution or a 1:30,000 dilution. The optimal dilutions were then repeated for their specificity of detection against each of the three proteins NP, VP35 and VP30. For these experiments, a 1:2000 dilution of anti-NP2 was used and 1:4000 dilutions of anti-VP30 and anti-VP35 were used. A secondary goat anti-mouse HRP antibody (KPL) was used at a 1:30,000 dilution in all cases.

#### 2.5.1.2.4 Analysis of RNP Complex Protein-Protein Interactions

Once protein expression from pCAGGS constructs could be confirmed, it was of interest to determine the intracellular localizations of the various REBOV proteins and whether, upon coexpression, the proteins would demonstrate protein-protein interaction and subsequent relocalization consistent with a previously reported study using MARV (Becker et al., 1998). To do this, RNP complex component expression plasmids were cotransfected into 293T cells seeded in LABTEK slides in the following 2-plasmid combination: NP + VP35, NP + VP30, VP35 + L, VP30 + L, and the localization of the proteins examined using the antibodies as described above. Anti- NP2 was used in a

1:400 dilution, anti VP35 in a 1:100 dilution and anti-VP30 in a 1:1000 dilution. All detection was with a secondary goat anti-mouse Alexa 488 antibody at a 1:200 dilution (Molecular Probes). In addition, combinations of RNP components from REBOV, ZEBOV and MARV were also coexpressed and their ability to interact determined based on protein relocalization in this system. REBOV VP35 and VP30 were detected, as described above, while MARV VP35 was detected with a mouse monoclonal antibody at a 1:1000 dilution and MARV VP30 was detected with a 1:100 dilution of an anti-MARV VP30 guinea pig serum. The mouse anti-MARV VP35 was detected with a secondary goat anti-mouse Alexa 488 antibody at a 1:200 dilution (Molecular Probes), while the guinea pig anti-MARV VP35 was detected with a rabbit anti-guinea pig serum at at a 1:300 dilution (Sigma). Fluorescent signals were viewed using an Axiovert 200M microscope (Carl Zeiss Canada Ltd.) and documented using an AxioCam HRm, colour video camera and the AxioVision imaging software package (Carl Zeiss Canada Ltd.).

#### 2.5.1.2.5 Analysis of RNP Complex Function in the ZEBOV Infectious Clone System

In order to confirm biological function of the pCAGGS expression constructs encoding the REBOV RNP components, they were transfected together with 1 μg of a ZEBOV full-length genome encoding plasmid (Neumann et al., 2002) into $5 \times 10^5$ cells of a 1:1 293T/VeroE6 mixture by Steven Theriault, as previous described (Neumann et al., 2002). The masses of REBOV helper plasmids transfected were as follows: 1 μg NP, 0.5μg VP35, 0.3 μg VP30 and 1 μg L. In addition, single or pair wise substitutions of REBOV RNP expression plasmids for their ZEBOV counterparts were made in the

ZEBOV infectious clone system. In all cases the plasmid masses above were used. Three days post-transfection, the supernatants were collected and used to infect fresh VeroE6 cells. These cells were monitored for the formation of CPE for 14 days. To confirm infection, VeroE6 cells were grown on cover slips and infected with supernatants generated above. Following an incubation of 4 days, the infected cells were fixed and inactivated with 2% (w/v) paraformaldehyde in PBS for 2 days with one fixative exchange. For immunofluorescence analyses, the following protocol was applied. Briefly, cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 15 min, washed three times with PBS, incubated with a 1:200 dilution of polyclonal rabbit serum directed against ZEBOV VP40 for 1 h at 37 °C, washed three times with PBS, incubated with a 1:250 dilution of FITC-labeled goat anti-rabbit conjugate (Sigma) for 1 h at 37 °C, and washed three times with PBS. The cover slips were mounted on glass slides and analyzed for fluorescence using an Axioplan 2 Fluorescence microscope (Carl Zeiss Canada Ltd).

### 2.5.1.3 Transfection

#### 2.5.1.3.1 Helper Plasmid-driven Minigenome System

293T cells were seeded into poly-D lysine coated 6 well tissue culture plates (Costar) and transfected with the various Pol I or T7-driven minigenome constructs along with helper plasmids encoding the RNP-complex proteins, using TransIT-LT1 (Mirus) as described above. In the case of the T7-driven minigenome system, 1 μg of a T7 polymerase encoding pCAGGS construct was also supplied (kindly provided by Dr. Yoshihiro Kawaoka, University of Wisconsin) by the same method. Additional samples were run in which VP30 and L were omitted in order to serve as controls for the

dependence of transcription and/or replication on the viral RNP complex. In these samples empty pCAGGS was supplied in order to maintain a constant mass of plasmid being transfected relative to samples in which all RNP components were supplied.

To determine the optimal mass of minigenome DNA to be transfected for each system, a range of minigenome masses from 0.125 μg to 2 μg was transfected along with standard amounts of helper plasmid. The masses used in these initial experiments corresponded to those found to be optimal during the establishment of the ZEBOV minigenome system (Muhlberger et al., 1999). After optimal amounts of minigenome were established, the transfected mass of minigenome DNA was held constant while the amounts of each transfected helper plasmid was increased and decreased up to 4 fold. All subsequent experiments were carried out using these optimized amounts of helper plasmid and minigenome DNA.

For all helper plasmid-driven experiments the transfected samples were incubated for 72 h prior to CAT assay, as described below.

#### 2.5.1.3.2 Infection-Driven Minigenome System

293T cells in poly-D lysine coated 10 $cm^2$ dishes (Costar) were transfected with various amounts of minigenome DNA between 0.25μg and 1 μg, as described above. These cells were then incubated at 37°C for 24 h prior to infection with REBOV at multiplicities of infection (MOIs) from 0.001 to 0.1 pfu/cell. Briefly, 2 mL of virus dilutions in DMEM without FCS and antibiotics were incubated with minigenome-transfected cells for 1 h at 37 °C. Following this incubation, the virus was removed and 6 mL of fresh DMEM (with 2 % (v/v) FCS and antibiotics) were added. Cultures were

incubated for a further 72 h at 37°C with 5 % $CO_2$ prior to harvesting for CAT assay as described below.

#### 2.5.1.4 Passaging of Recombinant REBOV

293T cells were transfected with 0.5 μg of 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. Cells were assayed for CAT activity, as described below, at 72 h post-infection, and the corresponding supernatants were passaged to fresh cell monolayers. To do this, debris was removed from the supernatants by centrifugation at 3000 x *g* for 10 min. Subsequently, 2 mL (out of 4mL total) of the undiluted supernatant were used to infect VeroE6 cells (approximately $10^6$) (passage 1). After 1 h of incubation at 37 °C the inoculum was replaced with fresh DMEM (with 2 % (v/v) FCS, antibiotics) and cells were incubated for a further 72 h at 37 °C. This process was repeated twice (passages 2 and 3).

### 2.5.2 ZEBOV Minigenome System

#### 2.5.2.1 Transfection

The development of a T7-driven CAT ZEBOV minigenome system has been previously described (Muhlberger et al., 1999). Both this construct and a similar Luciferase expressing construct were kindly provided to us through collaboration with Dr. Stephan Becker (Philipps Universität Marburg) and the system was set up based on this previously published protocol. Briefly, 1μg of ZEBOV minigenome plasmid along

with 1μg pCAGGS-NP, 0.5μg pCAGGS-VP35, pCAGGS-0.3μg VP30 and 1μg pCAGGS-L, as well as pCAGGS-T7, were transfected into 293T cells seeded in poly-D Lysine coated 6 well plates (Costar) using TransIT-LT1 (Mirus) as described above. Samples were incubated at 37°C for 48 h prior to harvest and CAT assay as described below.

### 2.5.3 MARV Minigenome Systems

#### 2.5.3.1 Transfection

While a T7-driven MARV minigenome has been previously published (Muhlberger et al., 1998), this construct was not available to us. Therefore, a T7-driven MARV minigenome and RNP expression plasmids based on this design were developed by our group and kindly provided for these experiments by Steven Theriault and Gülsah Mehmetoglu. This construct, along with the MARV RNP complex expression plasmids, was used to establish a minigenome assay based on the conditions previously determined to be optimal for MARV (Muhlberger et al., 1998, Muhlberger et al., 1999). In order to establish this system, 0.5 μg of MARV helper plasmid was transfected along with, 1μg of pCAGGS-NP, 0.5μg pCAGGS-VP35, pCAGGS-0.3μg VP30 and 1μg pCAGGS-L, as well as 1 μg pCAGGS-T7, into 293T cells seeded in poly-D Lysine coated 6 well plates (Costar) using TransIT-LT1 (Mirus), as described above. Samples were incubated at 37°C for 48 h prior to harvest and CAT assay as described below.

## 2.5.4 Reporter Assays

### 2.5.4.1 CAT Assays

CAT activity was assayed using the commercially available FastCAT system (Molecular Probes Inc., Eugene, Oregon) according to the manufacturer's instructions. For all helper plasmid cotransfection experiments 5 µl of cell lysate was used, while for experiments in which RNP complex proteins were supplied by virus infection, 20 µl of cell lysate was used. Reactions were incubated for 2 h except where otherwise noted prior to separation by thin layer chromatography using silica gel plates and an 87:13 (v/v) (chloroform:methanol) aqueous phase. Results were documented by photography and/or evaluated using the ImageQuant software (Amersham Biosciences Inc.) for the Typhoon Phosphoimager (Amersham Biosciences Inc).

### 2.5.4.2 GFP Imaging and FACS Analysis

Cells transfected with GFP-containing reporter minigenomes were assessed for GFP expression using an Axiovert 200M microscope (Carl Zeiss Canada Ltd., Toronto, Ontario). Documentation was performed using an AxioCam HRm colour video camera and the AxioVision imaging software package (Carl Zeiss Canada Ltd., Toronto, Ontario). Alternatively, cells were scraped into 1 mL PBS, centrifuged at 3000 x *g* and resuspended in 2% (w/v) PFA in PBS. Cells were fixed for 15 min in this solution prior to analysis using a FACSCalibur cytometer. Data were collected from 10,000 cells and analyzed using CellQuest Pro software (BD Biosciences).

### 2.5.4.3 Luciferase Assays

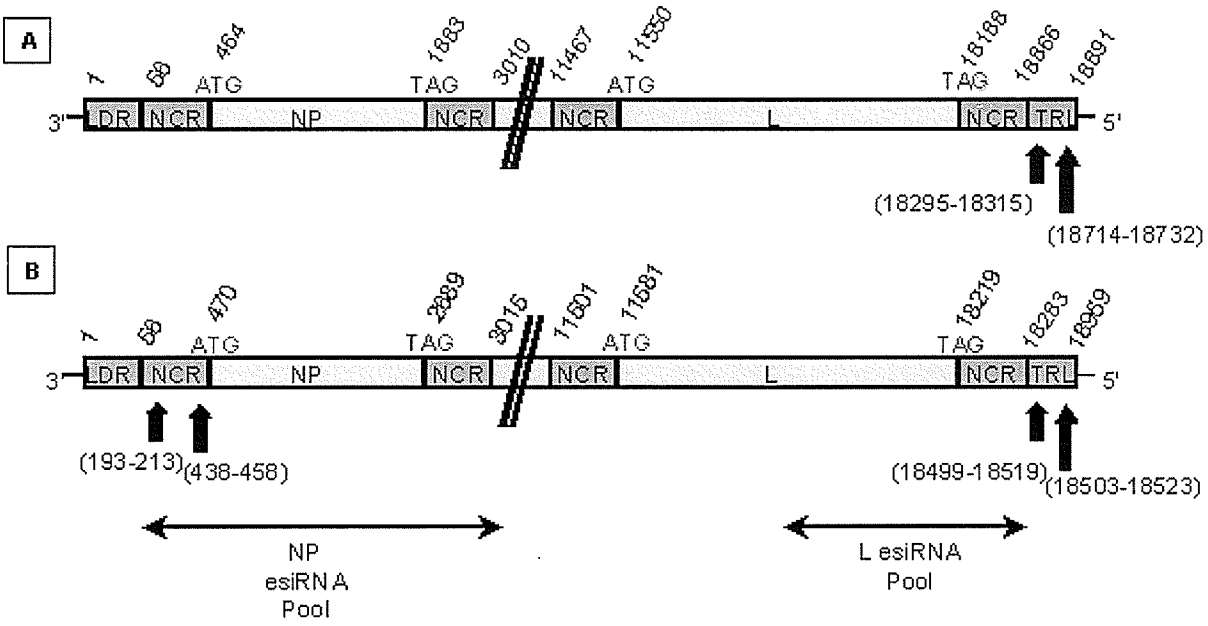
After incubation of transfected cells for 48hrs, samples were harvested by removing the supernatants and scraping cells into 1 mL PBS. Cells were centrifuged at 3500 x *g* for 10 min and resuspended in 200µl passive lysis buffer (Promega). For quantitation of luciferase activity, 20µl of lysate was placed in a white 96 well plate. Samples were then analyzed in a Veritas luminometer using the Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions. This kit makes use of an additional Pol II-driven Firefly expression construct, which is cotransfected to provide a signal for standardization based on the efficiency of transfection between samples.

## 2.6 *In vitro* siRNA Testing

### 2.6.1 siRNA Design

Potential siRNA target sites in the 3' and 5' non-coding and untranslated regions of the genome of ZEBOV (strain Mayinga) and REBOV (strain Pennsylvania) were identified using the siRNA Wizard, version 2.4 [www.sirnawizard.com] (Invivogen). Based on the top 10 "recommended" targets sites, non-overlapping sequences were selected which represented each of the target site-rich regions of the non-coding region (NCR) and untranslated region (UTR) sequences. The relative positions of identified siRNA target sites are shown in Figure 14. Oligonucleotides corresponding to the predicted target sites are listed in Table 12. Further, psiRNA-scramble, which contains an siRNA expression cassette that does not correspond to any known mouse or human genes (Invivogen), was used as a control. The target sequence for this shRNA is as follows: GCAUAUGUGCGUACCUAGCAU.

**Figure 14. EBOV-specific siRNA design.** In order to assess the potential of siRNAs to limit virus transcription and/or replication the Invivogen siRNA wizard prediction algorithm was used to identify favourable siRNA target sites within the 3' and 5' non-coding regions of the (A) REBOV and (B) ZEBOV genomes. Based on these predications siRNA molecules were designed complementary to the regions indicated by black arrows complementary to nucleotides 18295-18315 and 18713-18733 of the viral genome.




## 2.6.2 shRNA Cloning

Single-stranded oligonucleotides corresponding to the predicted siRNA target sites were synthesized by Operon Biotechnologies and had the basic structure: 5' *Bbs*I overhang – sense target sequence – loop (TCAAGAG) - antisense target sequence- 3' *Bbs* overhang. These shRNA expression cassette-encoding oligonucleotides were subsequently cloned into the psiRNA vector (Invivogen) under the control of the RNA Pol III promoter (Figure 15).

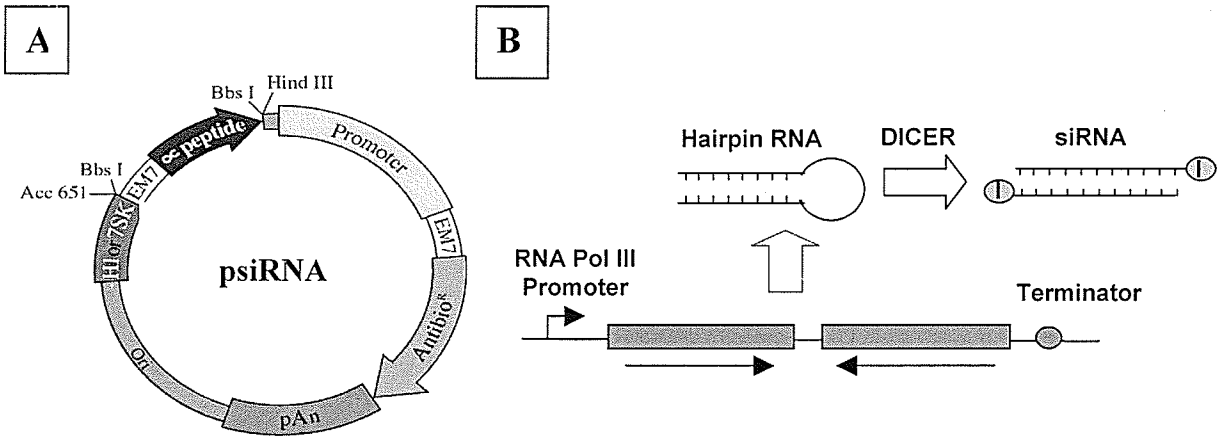
For cloning, the oligonucleotides were first phosphorylated for 1 h at 37°C using T4 DNA polynucleotide kinase (PNK) (NEB). Following phosphorylation,

**Table 12. shRNA encoding oligonucleotides and their corresponding target sites**

| Oligo Name | Target Site | Oligonucleotide Sequence |
|---|---|---|
| RL1 | REBOV L mRNA (nt 18295-18315) | 5'-ACCTCAGAGGTAACAAGCTATTAGTTCAAGAG AACTAATAGCTTGTTACCTCTTT |
| RL2 | | 5'-CAAAAAAGAGGTAACAAGCTATTAGTTCTCTT GAAACTAATAGCTTGTTACCTCTG |
| RL3 | REBOV L mRNA (nt 18714-18732) | 5'- ACCTCAACTCGATATGACCTCAAGTCTCAAG AGGACTTGAGGTCATATCGAGTTT |
| RL4 | | 5'-CAAAAAAACTCGATATGACCTCAAGTCCTCT TGAGACTTGAGGTCATATCGAGTTG |
| ZNP1 | ZEBOV NP 3' UTR (nt 193-213) | 5'-ACCTCGGCATCAGTGTGCTCAGTTGATCAA GAGTCAACTGAGCACACTGATGCCTT |
| ZNP2 | | 5'-CAAAAAGGCATCAGTGTGCTCAGTTGACT CTTGATCAACTGAGCACACTGATGCCG |
| ZNP3 | Zaire NP 3' UTR (nt 438-458) | 5'-ACCTCGACAAATTGCTCGGAATCACATCA AGAGTGTGATTCCGAGCAATTTGTCTT |
| ZNP4 | | 5'-CAAAAAGACAAATTGCTCGGAATCACACT CTTGATGTGATTCCGAGCAATTTGTCG |
| ZL1 | Zaire L mRNA (nt 18499-18519) | 5'-ACCTCGGGCTCATATTGTTATTGATATCAA GAGTATCAATAACAATATGAGCCCTT |
| ZL2 | | 5'-CAAAAAGTCTGGGCTCATATTGTTATTCTC TTGAAATAACAATATGAGCCCAGACG |
| ZL3 | Zaire L 5' UTR (nt 18503-18523) | 5'-ACCTCGGGCTCATATTGTTATTGATATCAA GAGTATCAATAACAATATGAGCCCTT |
| ZL4 | | 5'-GGGCTCATATTGTTATTGATACTCTTGATA TCAATAACAATATGAGCCCG |

oligonucleotide pairs were hybridized by heating to 100°C for 5 min in a Dry Bath Incubator (Fisher), after which the heating was turned off and the primers allowed to cool to RT slowly in the block. The psiRNA-hH1neo vector was cleaved using *Bbs*I (NEB)

**Figure 15. Expression strategy for REBOV-specific siRNA molecules. (A) The psiRNA shRNA expression vector.** All oligonucleotides for shRNA expression were cloned under the control of the hH7 RNA polymerase III promoter in a neomycin resistant verion of psiRNA. **(B) shRNA expression from psiRNA.** Following transfection into eukaryotic cells transcription of the above constructs yielded small hairpin RNA (shRNA) structure as a result of base pairing between the sense and antisense target-specific sequences. This structure is recognized and further processed by DICER to yield a conventional siRNA molecule capable of gene silencing.




and the resulting band purified using gel extraction (Qiagen). The vector was then dephosphorylated overnight at 37°C using shrimp alkaline phosphatase (Roche) and further purified using the PCR purification kit (Qiagen) prior to use. For cloning, the hybridized oligonucleotides preparations were serially diluted 1:3 in sterile water and mixed with a range of vector masses. Samples were ligated overnight at 14°C and then transformed into competent GT116 *E. coli* (Invivogen). Following plating on LB agar with 50 μg/mL kanamycin and growth overnight at 37°C, plasmid was prepared from resulting colonies and screened for oligonucleotides incorporation by restriction digest with *Cla*I and *Hind*III (NEB). Plasmids containing the oligonucleotides were verified by sequencing and prepared in larger amounts using a Maxiprep kit (Qiagen).

### 2.6.3 siRNA Screening Using the Minigenome Systems

In order to assess the ability of shRNA molecules to inhibit reporter gene expression from minigenome constructs, various amounts of the shRNA expressing plasmids, ranging from 0.25 μg to 2 μg, were transfected along with the necessary minigenome system components. For transfection, 6 well plates (Costar) were treated with Poly-D-Lysine and seeded for ~50% confluence 24 h later with 293T cells. The next day cells were transfected with the shRNA-expressing plasmids along with minigenome components. For the ZEBOV minigenome system 1μg of ZEBOV Luciferase minigenome along with 1μg of pCAGGS-NP, 0.5μg of pCAGGS-VP35, 0.3μg of pCAGGS-VP30, 1μg of pCAGGS-L and 1 μg of pCAGGS-T7 were used, while for REBOV the same amounts of helper plasmid were used, except for the omission of PCAGGS-T7. However, only 0.25 μg of Pol I CAT REBOV minigenome was transfected. Transfection was performed with TransIT-LT1 (Mirus) as described above and the cells were incubated for 48 h (ZEBOV) or 72 h (REBOV) prior to harvest.

### 2.6.4 Transient siRNA Transfection

#### 2.6.4.1 Lipid Transfection

293T cells and VeroE6 cells were transfected using TransIT-LT1 (Mirus) as described above. Into each well of 293T cells was transfected 1μg of psiRNA-ZNP1 or psiRNA-scramble, while for VeroE6 cells 1, 3 and 6 μg of each plasmid were used. After transfection the cells were allowed to recover by incubation at 37°C in the presence of 5% $CO_2$ overnight.

### 2.6.4.2 Electroporation

VeroE6 cells were prepared for electroporation as described above. Into individual cell preparations were electroporated 10 μg of either psiRNA-ZNP1, psiRNA-scramble or no DNA. After seeding into 6 well plates cells were allowed to recover by incubation at 37°C in the presence of 5% $CO_2$ for overnight.

## 2.6.5 Stable shRNA Expression

### 2.6.5.1 Electroporation of shRNA Plasmid

To generate stable cell lines, VeroE6 cells were prepared and 10 μg of psiRNA-ZNP1 was electroporated as described above. Following electroporation cells were seeded into a T25 flask (Costar) and allowed to recover during incubation at 37°C in the presence of 5% $CO_2$ for 24 h prior to being trypsinized and transferred to a T 75 (Costar) for antibiotic selection.

### 2.6.5.2 Antibiotic Selection

In order to establish an appropriate concentration of Geneticin at which to select plasmid containing cell clones, a killing curve was performed using the parent cell line VeroE6. To do this, VeroE6 cells were split for a 30% confluence 24 h later in 6 well plates. Twenty-four hours later, DMEM with 10% (v/v) FCS containing various amounts of Geneticin from 0-1000 μg/μl was added. Cells were incubated under these conditions for 10d with fresh selective media added every 3 days. Wells were assessed for the percentage of cell death and the concentrations producing 50% and 90% cell death after

10 days determined by visual inspection.

Based on these values, cell cultures into which the ZNP1 siRNA construct had been electroporated were incubated in media containing 400 μg/μl Geneticin for 10 days prior to plating for the selection of cell clones.

### 2.6.5.3 Cell Clone Propagation

For the selection of individual cell clones, electoporated cultures were split from 1:30 to 1:1000 into 10 $cm^2$ in order to obtain single cell foci. These foci were allowed to grow until they were ~100 cells in size. At this time 24 cell foci were picked by scraping and aspiration of the foci under a microscope using a pipette tip. These foci were then ejected into fresh media with Geneticin in a 24 well dish and allowed to grow to confluence at 37°C. During this time media was changed every 3-4 days in order to maintain antibiotic selection.

### 2.6.5.4 Cell Clone Screening

Selected cell clones were assessed for expression of the transfected siRNA based on the inhibition of minigenome expression in these cells. In order to assay this, ZEBOV minigenome components, including a Renilla luciferase reporter plasmid, were transfected into each cell lines using TransIT-LT1 (Mirus), as described above. A Pol II-driven Firefly expression construct for standardization was also included. Following a 48 h, incubation lysates were harvested for luciferase assay and the relative activities of the cell lines determined. Based on these results, cell lines with promising ability to inhibit minigenome transcription/replication were selected for further testing.

### 2.6.6 Infection with ZEBOV-GFP

ZEBOV-GFP was rescued by Steven Theriault using the ZEBOV infectious clone system (Neumann et al., 2002) as previously described (Hoenen et al., 2006). Virus stocks at a titer of 5 x $10^5$ ffu/mL were diluted 1:5 and 1:50 in DMEM without FCS to produce a high MOI (MOI = 0.05) and a low MOI (MOI = 0.005) dose, respectively. Infections were performed either using VeroE6 cells or stable cell line variants prepared from this line grown in 6 well plates to 100% confluence. Prior to infection cells were washed once with DMEM without FCS. The media was then removed and 0.5 mL of either the high or low MOI virus dilution was applied. Plates were incubated for 1 h at 37°C with rocking every 15 minutes. After infection the inoculum was removed and the cells washed three times with DMEM without FCS. Subsequently, 2.5 mL of DMEM with 2% (v/v) FCS was added to each well and the cells were incubated at 37°C for 3-4 days.

#### 2.6.6.1 FACS Analysis

ZEBOV infected cells were fixed for FACS analysis using freshly prepared 4% (w/v) PFA. Following incubation, as indicated above, the cells were washed twice with 1 mL PBS and then 0.8 mL PBS with 0.04% (w/v) EDTA was added to each well and the samples were allowed to incubate at 37°C for 15 min. Cells were then suspended and added to 8 mL 4% (w/v) PFA and kept at 4°C overnight. These cells were then centrifuged at 3500xg for 10 min and resuspended in 5 mL fresh 4% (w/v) PFA. These samples were transferred to fresh tubes and incubated for 30 min prior to being removed from BSL 4. Following FACS analysis the percentage of fluoresncent cells in each

population was graphed. Representative data from 2 (transient expression) or 3 (stable expression) independent replicates is shown.

### 2.6.6.2 Autofluorescent Plaque Assay

Supernatants from ZEBOV-GFP infected VeroE6 or stable cells were serially diluted 1:10 from $10^0$ to $10^6$ and 50µl of each dilution was used to infect VeroE6 cells grown to confluence in a 96 well plate. Prior to infection, cells were washed once with DMEM without FCS. The media was then removed and the virus dilutions applied. Plates were incubated for 1 h at 37°C with rocking every 15 minutes. After infection the inoculum was removed and the cells washed three times with DMEM without FCS. Subsequently, 200µL of Eagle's minimal essential medium (EMEM) with 5% (v/v) FCS and 1.5% (w/v) carboxymethyl cellulose (CMC) was added to each well. Cells were incubated at 37°C for 5 days to allow for focus formation. Cells were then washed 4x with 200µl PBS to remove all traces of CMC prior to fixation with 400µl 10% (w/w) formalin per well. Plates were stored at 4°C overnight. The plates were then placed in sealable bags to which an excess of fresh 10% (w/w) formalin was added prior to removal from BSL4 for analysis. Samples were stored for an additional 24 h at 4°C prior to use. Cells infected with GFP-ZEBOV were then counted based on GFP expression using an Axiovert 200M microscope (Carl Zeiss Canada Ltd.). Documentation was performed using an AxioCam HRm colour video camera and the AxioVision imaging software package (Carl Zeiss Canada Ltd.). Viral titres following infection were also graphed and, from 5 replicate experiments, a representative data set is shown.

### 2.6.6.3 Western Blot for NP Expression

From cell samples fixed for FACS analysis, a 2.5 mL aliquot was centrifuged and resuspended in 15µl PBS. To this was added 5µl of 4x gel loading buffer with β-mercaptoethanol. Samples were run at 100V until fully separated, as indicated by the position of the markers, at which time the proteins were transferred to PVDF by wet electrophoretic transfer as described above. Following blotting the PVDF was cut in half between the 50 and 60 kD marker bands and prepared for western blotting as described above. For detection of NP a 1:100 dilution of the B1-C6-6 monoclonal anti-ZEBOV NP antibody (the kind gift of S. Becker, Philipps Universität Marburg) was used, while for detection of actin a 1:500 dilution of clone AC-40 mouse anti-actin (Sigma) was used. Both proteins were detected using a 1:50,000 dilution of goat anti-mouse (Jackson Immunoresearch Laboratories) secondary antibody and chemiluminescent detection as described above.

### 2.6.6.4 Analysis of CPE

Confluent VeroE6 or stable derivative cell lines were prepared in 6 well plates. Cells were infected with an MOI = 0, MOI = 0.05 or MOI = 0.005 of ZEBOV-GFP for 3 or 4 days as described above. After incubation the media was removed and the cells were fixed in 4 mL of 10% (w/w) formalin overnight at 4°C. An excess of fresh 10% (w/w) formalin was then added to heat sealable bags containing the plates, after which they were closed and removed from BSL4 for analysis. Samples were stored for an additional 24 h at 4°C prior to use.

### 2.6.7 Infection with REBOV

VeroE6 or derivative stable cell lines were grown in 6 well plates to 100% confluence. Virus stocks at a titer of 1.5 x $10^6$ ffu/mL were diluted in DMEM without FCS to produce a high MOI (MOI = 0.05) and a low MOI (MOI = 0.005) dose. Prior to infection cells were washed once with DMEM without FCS. The media was then removed and 500 µl of either the high or low MOI virus dilution was applied. Plates were incubated for 1 h at 37°C with rocking every 15 minutes. After infection the inoculum was removed and the cells washed three times with DMEM without FCS. Subsequently, 2.5 mL of DMEM with 2% (v/v) FCS was added to each well and the cells were incubated at 37°C for 5 days. Ten-fold serial dilutions of cell culture supernatants were then prepared in DMEM without FCS. Target VeroE6 cells grow to 100% confluence in 24 well plates were washed once with DMEM without FCS prior to the addition of 200µl of virus dilutions and incubation at 37°C for 1 h. Plates were rocked every 15 min during incubation after which the inoculum was removed and 4 mL EMEM with 5% (v/v) FCS and 1.5% (w/v) carboxymethyl cellulose (CMC) was added to each well. Cells were incubated for 5 days to allow for focus development, after which they were washed 4x with 200µl PBS to remove all traces of CMC. Samples were fixed with 4 mL 10% (w/w) formalin per well and stored at 4°C overnight. Subsequently an excess of fresh 10% (w/w) formalin was added to the plates in sealable bags, which were then closed and removed from BSL4 for analysis. Samples were stored for an additional 24 h at 4°C prior to use.

### 2.6.7.1 IFA for REBOV VP30 Expression

Plates were washed three times with PBS and then permeabilized in PBS with 0.1% (v/v) Triton X-100 for 15 min. Cells were washed a further three times with PBS prior to incubation at RT for 1 h with a 1:1000 dilution of an anti-VP30 mouse polyclonal antiserum. Cells were washed three times with PBS and incubated for 1 h at RT with a 1:200 dilution of goat anti-mouse Alexa 488 (Molecular Probes). Cells were washed a final three times with PBS prior to counting of the foci. This experiment was conducted twice and a representative data set is shown for REBOV titres after infection.

## 2.7 *In vivo* siRNA Testing

### 2.7.1 Formulation of Plasmid-PEI Complexes

Animals were to be treated with *in vivo* jetPEI-Gal (galactose conjugated for increased liver targeting) or *in vivo* jetPEI-Man (mannose conjugated for increased macrophage and dendritic cell uptake) complexes containing 50μg of either psiRNA-ZNP1, psiRNA-scrambled or the PEI complex alone. In order for PEI complexes to be correctly formed and bind the cell surface appropriately, the ratio of positive nitrogen residues in the PEI should be between 5 and 10 fold greater that of the number of negative phosphates on the DNA it is encapsidating (i.e. N: P = 5-10). Therefore in order to calculate the amount of PEI needed for the 50μg of DNA to be delivered the following calculation, supplied by the manufacturer (Polyplus Transfection), was applied:

$$\mu g\ PEI\ required = [(\mu g\ DNA \times 3\ nmol\ P^{-}/\mu g\ DNA) \times (N{:}P\ ratio)] / (150\ nmol\ N^{+}/\mu l\ PEI)$$

For a 50 μg DNA dose this calculation yields an input volume of PEI of 5 μl per dose. To prepare the complexes 50μg of endo-toxin-free DNA was diluted into 50 μl of a 10% (w/v) glucose solution. To this was added 50 μl of sterile RNase/DNase-free apyrogenic water. The mixture was then vortexed gently and centrifuged briefly to collect the material. Separately, 5μl of PEI was diluted into 50 μl 10% (w/v) glucose. To this was added 45 μl of sterile RNase/DNase-free apyrogenic water. The mixture was vortexed briefly and centrifuged to collect the material. The diluted PEI mixture was then added all at once to the DNA solution and the resulting mixture vortexed and centrifuged briefly. The PEI/DNA mixture was allowed to incubate for 15 min at room temperature before it was ready for use.

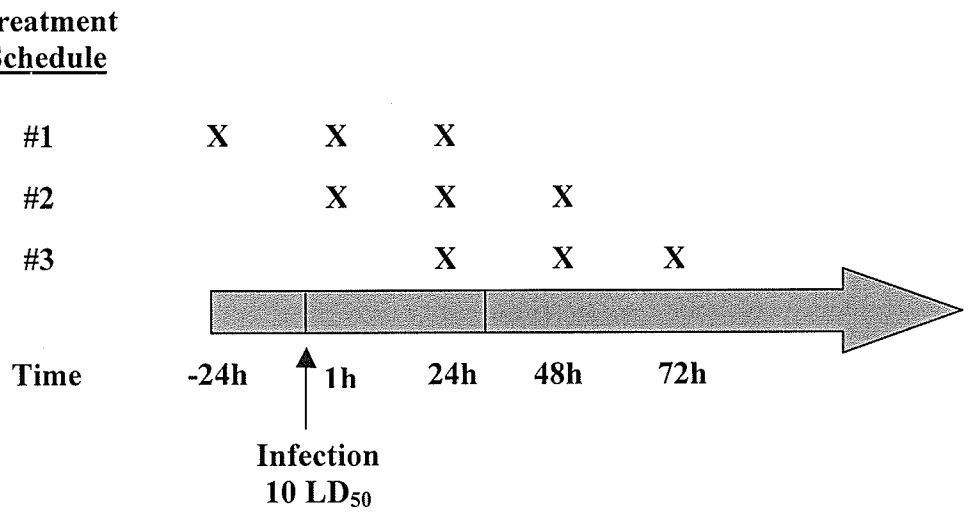
### 2.7.2 siRNA Treatment of BALB/c Mice

Following PEI complex formulation, complexes were transported into BSL-4 for same day use. Animal groups were injected with PEI complexes either 24 h before challenge, 1 h post-infection or 24 h post-infection. In all cases PEI complex treatments were continued every 24 h for a total of 72 hours. PEI complexes tested were those described above and at each time point 100μl of PEI complex in a 5% (w/v) glucose solution was given intra-peritoneal (i.p.) at 2 distinct sites. An experimental summary of the groups is described in Table 13 and a timeline of treatment is shown in Figure 16.

**Table 13. Experimental set up of shRNA treatments groups**

| PEI Delivery System | Time of Treatment | | |
|---|---|---|---|
| | **24 h pre-infection** | **1 h post-infection** | **24 h post-infection** |
| *in vivo*-jetPEI-Gal | ZNP1 shRNA<br>Scrambled shRNA | ZNP1 shRNA<br>Scrambled shRNA | ZNP1 shRNA<br>Scrambled shRNA |
| *in vivo*-jetPEI-Man | ZNP1 shRNA<br>Scrambled shRNA | ZNP1 shRNA<br>Scrambled shRNA | ZNP1 shRNA Scrambled shRNA |
| None | ZNP1 shRNA | ZNP1 shRNA | ZNP1 shRNA |

**Figure 16 Timeline of siRNA-PEI complex treatment.** Animals were given intra-peritoneal injections according to one of three treatment schemes. Accordingly, dosing began 24 h pre-infection, 1 h post-infection or 24 h post-infection. In each case treatment was continued daily until three doses of treatment had been given to each group.




## 2.7.3 Infection of Mice with MA-ZEBOV

Five to six-week-old female BALB/c mice were obtained from a commercial supplier (Charles River Laboratories). All mice were housed in microisolator cages and

allowed to acclimatize for 5 days prior to use in experiments. To assay for improved outcome, 16 groups of 5 mice were inoculated intraperitonially (i.p.) at two distinct sites with 10 LD50s of mouse-adapted ZEBOV (Bray et al., 1998) in 100 µl of DMEM. Two distinct injection sites were used to decrease the risk that virus was delivered into an undesirable location in the abdomen (i.e. bladder or intestine, rather than the peritoneal cavity). These animal experiments were performed on animal use documents approved by the Canadian Science Centre for Human and Animal Health - Animal Care Committee according to the guidelines of the Canadian Council on Animal Care

### 2.7.4 Clinical Monitoring

Following injection, mice were observed for clinical symptoms and weighed daily for 15 days. Surviving animals were observed for at least 28 days (three times longer than the time to death for fatally infected control animals) to ensure that recovery was complete.

### 2.7.5 Data Analysis

The weights for all infected animals were converted to a percentage of starting weight and all plotted as change in weight over time using Microsoft Excel (Microsoft Corp.). Based on these data graphs were also prepared indicating the number of animal which lost more than 5% or 10% of their body weight.

# 3.0 Results

## 3.1 Genome Sequencing

In order to obtain the basic molecular information required by our future goals, it was first necessary to obtain the full-length genomic sequence information for REBOV. In order to begin the sequence determination for this virus we used previously published Reston-specific diagnostic primers (Sanchez & Feldmann, 1996, Sanchez et al., 1996) [Appendix A]. From this starting point, regions of the genome were amplified and sequenced in order to provide data for new primers. Use of this "primer walking" approach lead to the sequencing of the complete sequence, except the extreme 3' and 5' termini. In order to obtain this sequence the viral genome was circularized and the termini then amplified by PCR. The result of this work was the compilation of the complete genome sequence for REBOV (strain Pennsylvania) [Appendix C], which is archived in GenBank under Accession Number AF522874.

### 3.1.1 Genome-Wide Comparison

With the complete genome sequence of REBOV available to us, it was of interest to determine whether there were any major alternations in the genome structure which might account for its altered virulence, compared to other filoviruses. However, sequence analysis (Tatusova & Madden, 1999) indicated that REBOV has the same gene order as its closest relatives, ZEBOV (Sanchez et al., 1993, Sanchez et al., 1996, Volchkov et al., 1999) and SEBOV (Sanchez & Rollin, 2005), as well as the more distantly related MARV (Bukreyev et al., 1995, Feldmann et al., 1992) (Figure 17A). Based on genome-wide comparisons of the available filovirus genomes it was observed

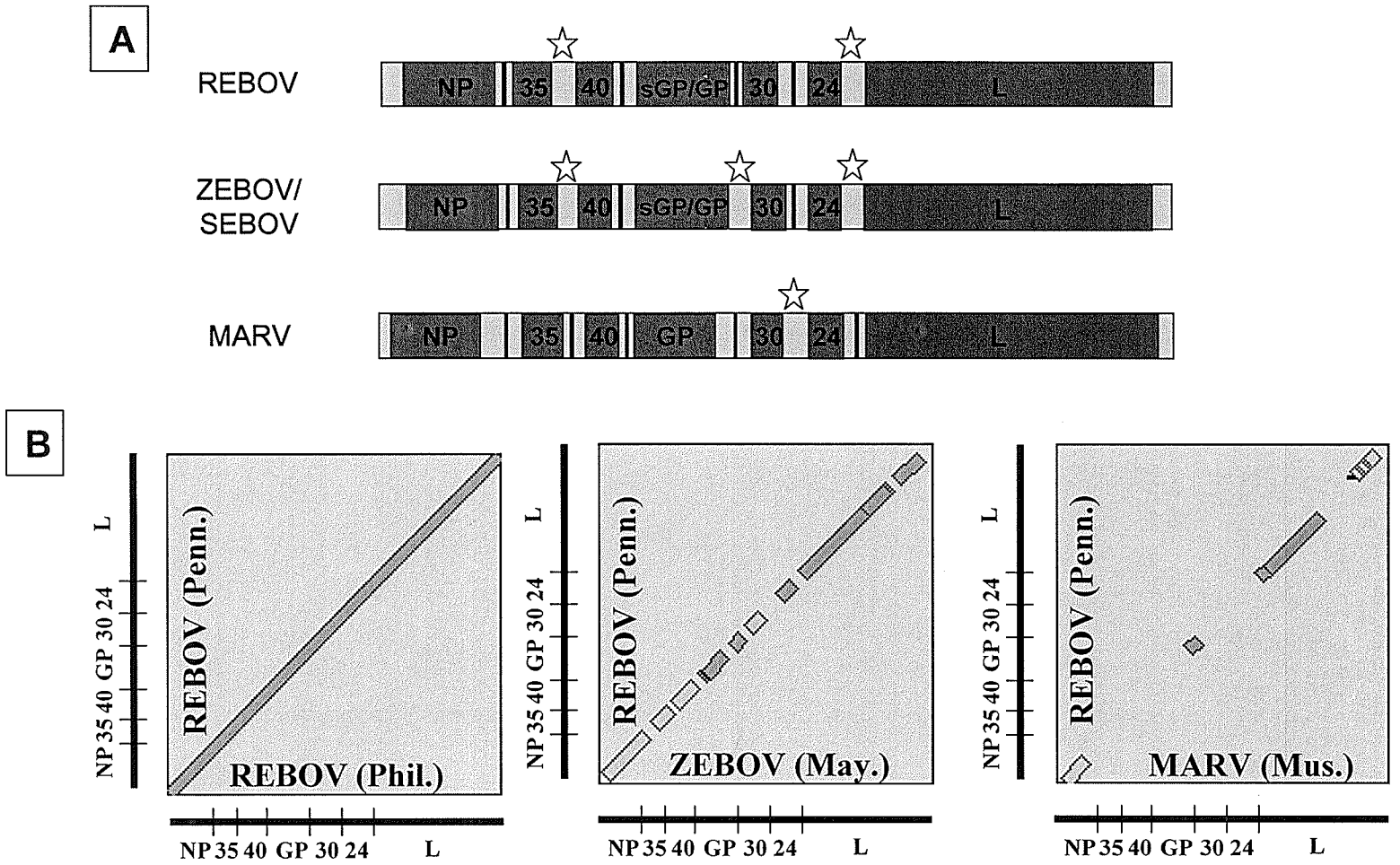



**Figure 17. Genome organization and homology of filoviruses (A) Filovirus genome organization.** The gene order of REBOV in comparison to other fully sequenced filovirus genomes is presented. The intergenic regions are shown in black, the non-coding regions in gray and the open reading frames in red. Stars indicate the positions of the gene overlaps. **(B) Genome-wide nucleotide comparison.** Graphical representations of genome-wide BLAST comparisons Between REBOV (strain Pennsylvania) (Accession #AF522874) and other fully sequenced filoviruses, including REBOV (strain Philippines) (Accession #AB050936), ZEBOV ((Accession #AF272001) and MARV (Z12132. Strong matches are shown in dark blue while weaker matches are shown in light blue. Regions of low homology are shown as gaps.

that the Pennsylvania strain of REBOV shows a high degree of homology to a concomitantly analyzed Philippine strain of REBOV (Ikegami et al., 2001). As expected, both the coding regions and the non-coding regions, including intergenic regions, were almost completely conserved. When compared to ZEBOV (strain Mayinga), conservation could be found within all the open reading frames, as well as the 3' leader and 5' trailer regions (Figure 17B). Surprisingly, the non-coding and intergenic regions did not show any homology between REBOV and ZEBOV. In contrast, comparison to MARV showed only shorter regions of conservation within some of the protein open-reading frames, particularly NP, GP and L, in addition to conservation of the genome termini. These nucleotide sequence comparisons of the Pennsylvania strain with the Philippine strain of EBOV Reston, EBOV Zaire (strain Mayinga) and MBGV (strain Musoke) clearly demonstrated the relationship of the different viruses within the family *Filoviridae* and the genus *Ebolavirus* (Figure 17B). They also confirmed previous phylogenetic analyses based on the open reading frame of the glycoprotein gene (Sanchez et al., 1996).

### 3.1.2 Comparison of Non-coding Regions

As with other filoviruses, the transcriptional start and stop signals were found to be highly conserved (Table 14). These signals were identical for the two strains of REBOV and, in comparison to ZEBOV, differences were found only in the most 5' base of the proposed transcriptional stop signal of the NP, VP30 and VP40 genes (Table 14). While the stop signals of NP and VP40 of REBOV contained only 5 uridine residues (6 in ZEBOV), the signal in VP30 was longer by a single uridine residue (5 in ZEBOV).

| Gene | Virus | Start Site (3'-5') | Stop Site (3'-5') |
|---|---|---|---|
| NP | Reston | CUCCUUCUAAUU | UAAUUCUUUUUG |
| | Zaire | CUCCUUCUAAUU | UAAUUCUUUUUU |
| VP35 | Reston | CUACUUCUAAUU | UAAUUCUUUUU |
| | Zaire | CUACUUCUAAUU | UAAUUCUUUUU |
| VP40 | Reston | CUACUUCUAAUU | UAAUUCUUUUUC |
| | Zaire | CUACUUCUAAUU | UAAUUCUUUUUU |
| GP | Reston | CUACUUCUAAUU | UAAUUCUUUUU |
| | Zaire | CUACUUCUAAUU | UAAUUCUUUUU |
| VP30 | Reston | CUACUUCUAAUU | UAAUUCUUUUUU |
| | Zaire | CUACUUCUAAUU | UAAUUCUUUUUG |
| VP24 | Reston | CUACUUCUAAUU | UAAUUCUUUUUC |
| | Zaire | CUACUUCUAAUU | UAAUUCUUUUUU |
| | | | UAAUUCUUUUUG |
| L | Reston | CUACUUCUAAUU | UAAUAAAAAAA |
| | Zaire | CUACUUCUAAUU | UAAUAAAAAAA |

**Table 14. Comparison of the transcriptional start and stop signals of REBOV (strain Pennsylvania) and ZEBOV (strain Mayinga).** Conserved UAAUU pentamers are underlined and highlighted in green with the corresponding non-consensus AU-rich sequences in the L transcriptional stop signals shown in green only. Non-conserved final U residues in the transcriptional stop signals and their counterparts are shown in red. (Modified from Groseth et al., 2002)

The remaining REBOV and ZEBOV genes demonstrate only 5 uridine residues, making 3'-UAAUUCU$_{(5)}$-5' a more accurate consensus sequence for the transcriptional stop signal for all EBOV genes. A stretch of 5 uridine residues is also consistent with the transcriptional stop signals of the MARV genes (Feldmann et al., 1992). In addition, REBOV displays the same non-consensus transcriptional stop signal following the L open reading frame that is seen in the ZEBOV genome. However, REBOV lacked the second transcriptional stop signal following the VP24 coding region found in ZEBOV (Sanchez et al., 1993). It remains to be shown what effect this missing second transcriptional stop signal might have on virus transcription or replication. In addition, the positions of gene overlaps were conserved between REBOV and ZEBOV, with the exception of the GP-VP30 overlap, which was absent in REBOV where it was replaced with separate consensus transcriptional start and stop sequences. Interestingly, a high degree of homology was also observed between the 3' leader and 5' trailer regions of ZEBOV and REBOV, and even the more distantly related MARV (Figure 18). This clearly implies an important role for the genomic ends in transcription and replication of filoviruses.

### 3.1.3 Protein Open Reading Frame Analysis

Nucleotide sequence comparison of the open reading frames of ZEBOV and the Pennsylvania strain of REBOV revealed significant identity ranging from 59.4 – 69.0% (Table 15). This level of conservation is not surprising since the proteins of related viruses need to carry out similar functions in the life cycle. The lowest degree of homology was found in the open reading frame of gene 4, which encodes the structural

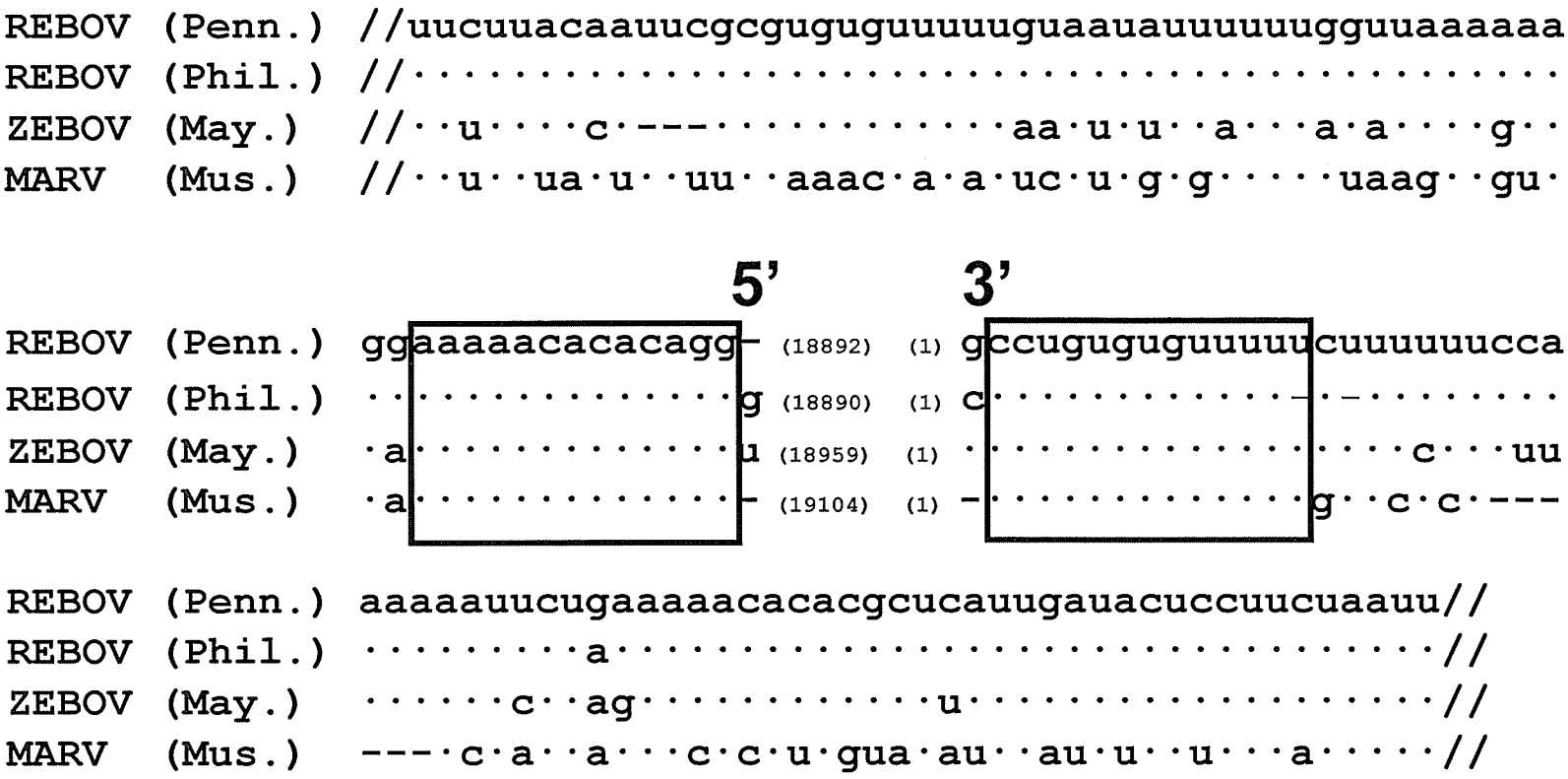



**Figure 18. Sequence comparison of filovirus genome termini.** Conservation of the extreme genome termini can clearly be seen not only between different strains of REBOV but also between EBOV species and even with MARV. Identical nucleotides are replaced dots for clarity and regions of complete conservation between all sequenced filoviruses are shown boxed. In addition, these conserved region showed perfect complementarity, supporting the hypothesis that interactions between these termini may occur, thereby facilitating their sequence conservation.

| | ZEBOV (May.) | | REBOV (Phil.) | |
|---|---|---|---|---|
| | nt (ident.) | aa (ident. / sim.) | nt (ident.) | aa (indent. /sim.) |
| NP | 67.1 | 68.6 / 80.9 | 98.9 | 98.6 / 98.8 |
| VP 35 | 66.2 | 64.7 / 76.8 | 98.1 | 98.8 / 99.7 |
| VP 40 | 67.0 | 74.0 / 82.8 | 98.9 | 98.5 / 98.8 |
| GP | 59.4 | 58.5 / 72.2 | 98.7 | 97.7 / 98.5 |
| sGP | 63.8 | 65.1 / 81.5 | 98.5 | 98.9 / 99.7 |
| VP 30 | 65.6 | 68.8 / 79.9 | 99.3 | 99.7 / 99.7 |
| VP 24 | 69.0 | 80.1 / 90.0 | 99.5 | 100 / 100 |
| L | 66.2 | 74.2 / 84.5 | 99.1 | 98.8 / 99.2 |

**Table 15. Nucleotide and amino acid comparisons between REBOV and ZEBOV protein open reading frames.** The percentage of identical nucleotide as well as the percentage of identical or similar and amino acids between REBOV(strain Pennsylvania), REBOV(strain Philippines) and ZEBOV(strain Mayinga) are shown. Values are given for each protein open reading frame, including the edited product of the GP gene, $GP_{1,2}$. The open reading frame with the highest homology is shown in green, while that with the lowest is shown in red.

glycoprotein. This was expected since the glycoprotein is the only surface antigen of viral particles and the target for neutralizing antibodies (Maruyama et al., 1999, Sullivan et al., 2000, Wilson et al., 2000). The highest percent identity was found in the VP24 open reading frame. The function of VP24 has not yet been determined, but it has been associated with the process of host adaptation (Volchkov et al., 2000)(H. Ebihara, personal communication). As expected, the two REBOV strains showed a high degree of homology and varied only by 0.5 – 1.9% in their open reading frame sequences (Table 15), a value that is in line with comparisons between different isolates of ZEBOV (Sanchez et al., 1996, Volchkov et al., 1997).

Comparison of the deduced amino acid sequences derived from the open reading frames of the different ZEBOV (strain Mayinga) and REBOV (strain Phennsylvania) genes revealed identities and similarities ranging from 58.5 – 80.1% and 72.2 – 90.0%, respectively (Table 15). Specifically, significant differences were found in the carboxyl-terminal half of the NP, a region which is less conserved among filoviruses and has been discussed for a potential role in the assembly process (Feldmann & Kiley, 1999), at the amino-terminal end of VP35, where the first 11 amino acids were missing in a direct comparison, and at the carboxyl-terminal end of VP40 where an extension of 5 amino acids (QNSYQ) was found. This extension of the VP40 open reading frame likely accounts for a previously demonstrated decrease in the SDS-PAGE mobility of this protein compared to SEBOV and ZEBOV (Feldmann et al., 1994). The glycoprotein was the least conserved protein with an identity and similarity of 58.5% and 72.2%, respectively. The soluble form sGP, which is the primary product of the glycoprotein gene (Sanchez et al., 1996, Volchkov et al., 1995) showed a higher conservation than the

GP. Differences were mainly found in the carboxyl-terminal half of the protein but also included a single amino acid insertion in the signal sequence, which is shared by sGP and GP. In VP30 most changes occurred at the carboxyl-terminus of the protein. The last 26 amino acids lacked substantial homology to ZEBOV and included a single amino acid truncation. The highest degree of identity and similarity were found with VP24 and L. This was expected for the L protein, since RNA-dependant RNA polymerases of filoviruses and other members of the order *Mononegavirales* display conservation (Muhlberger et al., 1992, Volchkov et al., 1999). In particular, the N-terminal half of the polymerase is well conserved even between the different filovirus genera (Figure 17B). This region contains a number of important functional elements including an RNA binding element, motif A, a putative RNA template recognition and/or phosphodiester bond formation domain, motif B, an ATP and/or purine ribonucleotide triphosphate-binding domain, motif C, as well as a pair of conserved cysteine residues. These features are present in all NNS L proteins. In contrast to the expectation of sequence conservation in L, the high degree of conservation in the VP24 was surprising. However, this may be in line with the role of this protein in host adaptation, as mentioned previously.

When the Pennsylvania and Philippine strains of REBOV were compared, there was between 97.7 - 100% identity and 98.5 - 100% similarity between the protein sequences (Table 15). In the majority of proteins these changes were scattered evenly throughout the coding region with two obvious exceptions. The changes in NP were again predominantly found in the less conserved carboxyl-terminal portion of the protein, while in the structural glycoprotein most of the changes occurred in the middle third, which is known to be the most variable part of this protein, carrying the majority of the

glycosylation sites for N and O-glycans (Feldmann & Kiley, 1999, Sanchez et al., 1998, Will et al., 1993).

## 3.2 Antibody Production

The availability of a substantial amount of sequence data made possible, for the first time, the production of antibodies against specific REBOV proteins. The production of such reagents to detect the expression of the RNP complex components, NP, VP35, VP30 and L, was a necessary next step in developing reverse genetics systems based on the functions of these viral proteins.

### 3.2.1 Expression and Purification of Viral Proteins and Peptides

In order to produce antibodies against the RNP proteins, a significant amount of the purified targets was required. For this purpose, the entire VP35 and VP30 open reading frames were cloned into the pGEX-6P #3 expression vector. Since the NP and L proteins were too large for expression in their entirety, peptides from each of these proteins were selected based on their hydrophobic and surface exposure characteristics. The sequences encoding these peptides were then cloned into pGEX-6P #1. Growth of BL21 *E. coli* containing these GST fusion protein expression plasmids under conditions of induction with IPTG lead to the expression of high levels of the fusion proteins. The expression of a representative pair of fusion proteins, VP35 and VP30, is show in Figure 19A. The presence of the GST moiety made purification using affinity chromatography possible, an approach that yielded sufficiently pure preparations for use in polyclonal antibody generation. A representative set of results from affinity purification for VP30

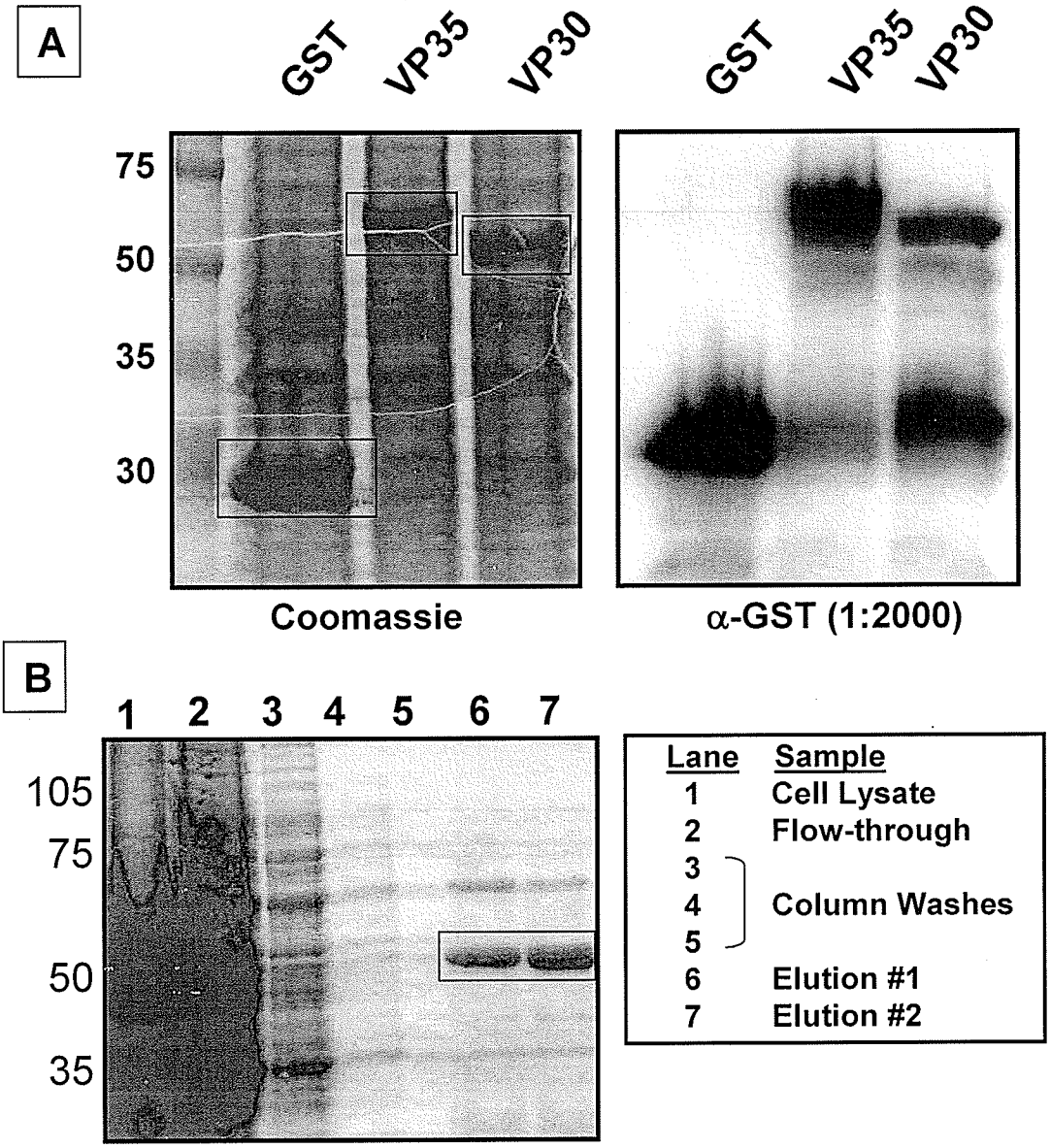



**Figure 19. Expression and purification of GST-VP35 and GST-VP30. (A) SDS-PAGE separation of bacterial cell lysates expressing VP35-GST and VP30-GST.** Cell lysates induced to express VP-35-GST, VP30-GST or GST alone by treatment with IPTG were separated on 10% polyacrylamide gels using SDS-PAGE. To examine the relative amount of GST or GST fusion protein expressed compared to total protein gels were stained with Commassie brilliant blue R-250. The VP30-GST and VP35-GST fusion proteins, we well as the GST proteins alone, are indicated by red boxes. Proteins from duplicate samples were transferred to PVDF for western blot using an anti-GST antibody in order to verify the identity of the protein products seen by Commassie staining. **(B) Affinity purification of GST-VP30.** In order to monitor the progress of affinity purification using glutathione-sepharose fractions were taken from the initial lysate, column flow-through, all wash steps, as well as the final elution fractions. All samples were run on 10% gels using SDS-PAGE and visualized for protein content by staining with Commassie brilliant blue R-250.

are shown in Figure 19B. However, since the preparations were not entirely homogeneous, in particular containing significant amounts of GST alone, estimation of protein content based on biochemical methodologies would have resulted in an overestimation of protein content. Therefore, assessment of the concentration of protein was based on visual estimation of band intensity in Commassie stained gels in comparison to commercial standards. Based on this comparison the protein content was estimated to be 0.2 μg/μl for VP35, 0.33 μg/μl for VP30 and both NP peptides, 1μg/μl for the L1 peptide and 2 μg/μl for the L2 peptide. These estimates were used to prepare 15 μg doses for immunization of 5-6 week old female Balb/c mice. Each mouse received one immunization and one booster shot prior to serum collection.

### 3.2.2 Analysis of Antibody Detection

Once sera were obtained from animals immunized with each of the fusion proteins it was necessary to determine whether they were capable of detecting the corresponding REBOV protein. For this purpose, RNA polymerase II eukaryotic expression plasmids encoding each of the RNP complex proteins were cloned using pCAGGS (Figure 20A). This vector system was chosen because its chicken β-actin derived promoter coupled with an SV40 origin of replication, which leads to replication of the plasmid in T antigen containing cells, allows for high levels of protein expression in 293T cells, a situation that is further enhanced by the highly transfectable nature of this cell type. Therefore, 293T cells were singularly transfected with each of the RNP expression constructs for IFA or, following harvest and separation by SDS-PAGE, for western blotting.

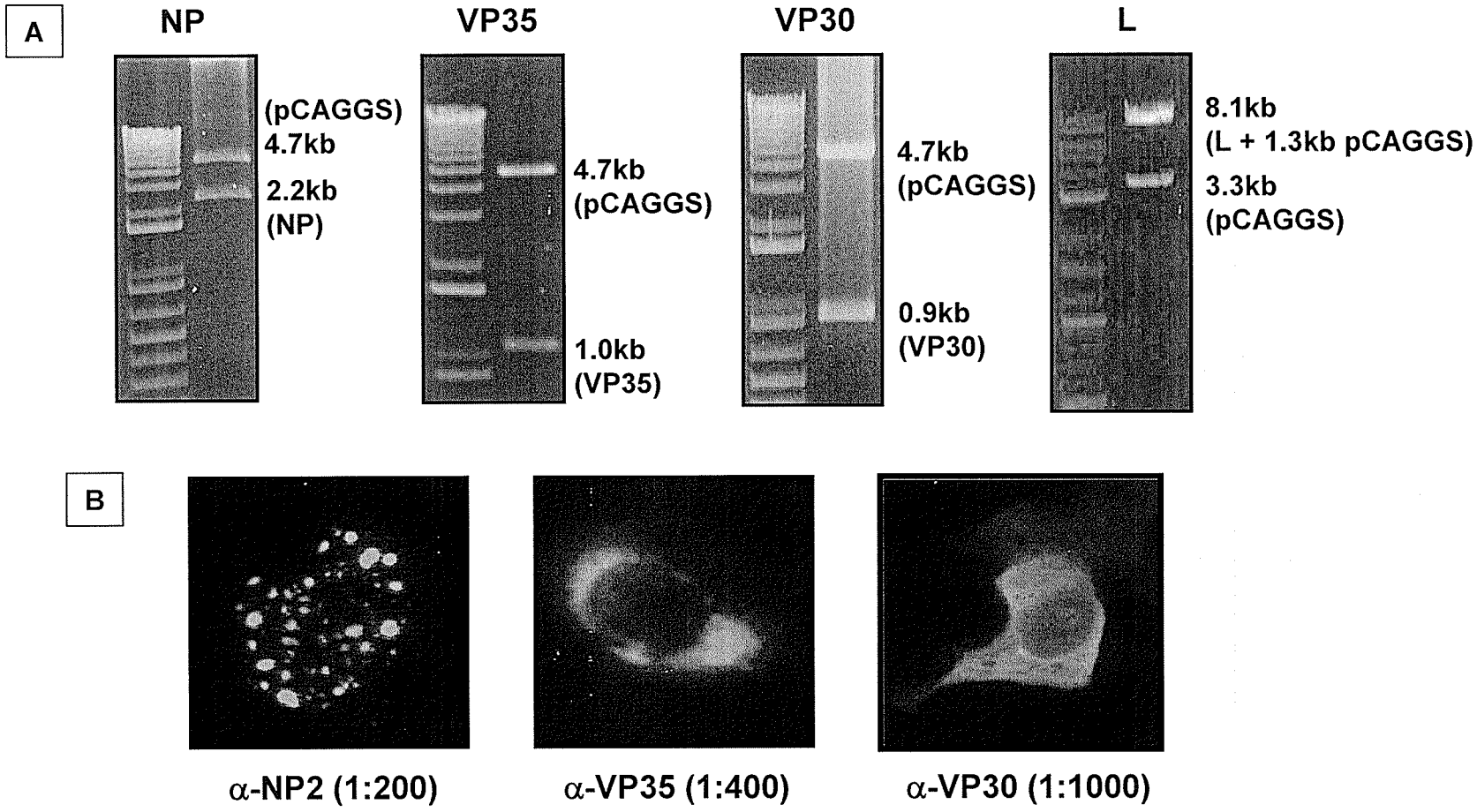



**Figure 20. Detection of REBOV RNP protein expression from pCAGGS constructs by IFA. (A) Cloning of pCAGGS constructs expressing REBOV RNP proteins.** Each of the RNP protein open reading frames were cloned into the pCAGGS multiple cloning site(MCS). Correct cloning of the inserts into the pCAGGS vector (4.7 kb) was confirmed by restriction analysis using *Eco*RI for NP (2.2 kb) and VP35 (1.0 kb), *Eco*RI and *Xho*I for VP30 (0.9 kb) and *Spe*I and *Bgl*II for L (8.1 kb). For restriction digest of L a vector site was chosen resulting in an increase in the size of the insert by ~1.4 kb and a corresponding decrease in the size of the vector. **(B) Visualization of RNP expression by IFA.** pCAGGS constructs expressing NP, VP35 and VP30 were transfected into 293T cells and expression detected using anti-NP peptide, anti-VP35 or anti-VP30 antisera, respectively. We were unsuccessful in detecting the L protein in transfected cells.

### 3.2.2.1 Detection by IFA

Following transfection into 293T cells for IFA it was possible to observe staining with the anti-NP2, anti-VP35 and VP30 antisera. Using a variety of antibody dilutions, it was found that optimal staining could be achieved using the following conditions: 1:200 anti-NP, 1:400 anti-VP35 and 1:1000 VP30.

Staining of the IFA samples indicated that NP is present in cytoplasmic inclusions, while both VP35 and VP30 were evenly dispersed throughout the cytoplasm (Figure 20B). These localizations are consistent with those previously reported for the corresponding MARV proteins (Becker et al., 1998). Unfortunately expression of L could not be directly confirmed. This could be due either to the lack of expression of L from the expression construct or low affinity of the antibodies. Since functional expression of L was late confirmed it is likely that the anti-L peptide antisera fail to detect the protein when expressed in its entirety, despite strong detection of the GST fusion peptides against which they were raised. This was not completely unexpected since a number of attempts have been made in the field to generate anti-L antisera but have been universally unsuccessful (H.Ebihara, personal communication; T. Hoenen, personal communication).

### 3.2.2.2 Detection by Western Blot

In order to further determine whether the antibodies capable of detecting the native viral proteins by IFA would also be sufficient to allow detection of the linear epitopes presented in western blotting, cell lysates from cells transfected with each of pCAGGS-NP, pCAGGS-VP35 and pCAGGS-VP30 were prepared and separated by

SDS-PAGE. Following transfer to a PVDF membrane, western blotting was performed with various dilutions of primary antibody, together with either a 1:10,000 or 1:30,000 dilution of secondary antibody. The anti-NP2, anti-VP35 and anti-VP30 polyclonal antisera were all found to detect proteins in western blot with the optimal concentrations for use in this application determined to be 1:2000, 1:4000 and 1:4000, respectively, with a 1:30,000 dilution of secondary goat anti-mouse antibody (KPL). Western Blots of protein detection for each of the three antisera are shown in Figure 21A. While the amino acid sequence against which the anti-NP2 peptide antiserum was raised is not conserved in ZEBOV, our sequencing data suggested that, based on their close relationship at the amino acid level, cross-species detection with the anti-VP35 and anti-VP30 antibodies should be possible. Accordingly, western blots were also performed against ZEBOV transfected cell lysates using various dilutions of the anti-VP35 and anti-VP30 sera. Optimal dilutions for this application were found to be 1:20 and 1:100 with a 1:50,000 dilution of secondary mouse anti-goat (Jackson Immunoresearch Laboratories) (Figure 21B). Thus, while these antisera demonstrate a high affinity for REBOV, allowing them to be used at high working dilutions, they are also have sufficient affinity for ZEBOV to allow detection at very low working dilutions. While less than ideal, these conditions allow application of these sera for detection of ZEBOV, for which specific anti-VP35 and VP30 antibodies are also rare.

In the detection of REBOV NP and both REBOV and ZEBOV VP35, a laddering of bands is observed. This may reflect the existence of various phosphorylation states of these proteins, although the extent to which VP35 is phospohorylated is controversial and seems to be cell line dependant. Alternatively, it is also possible that these bands

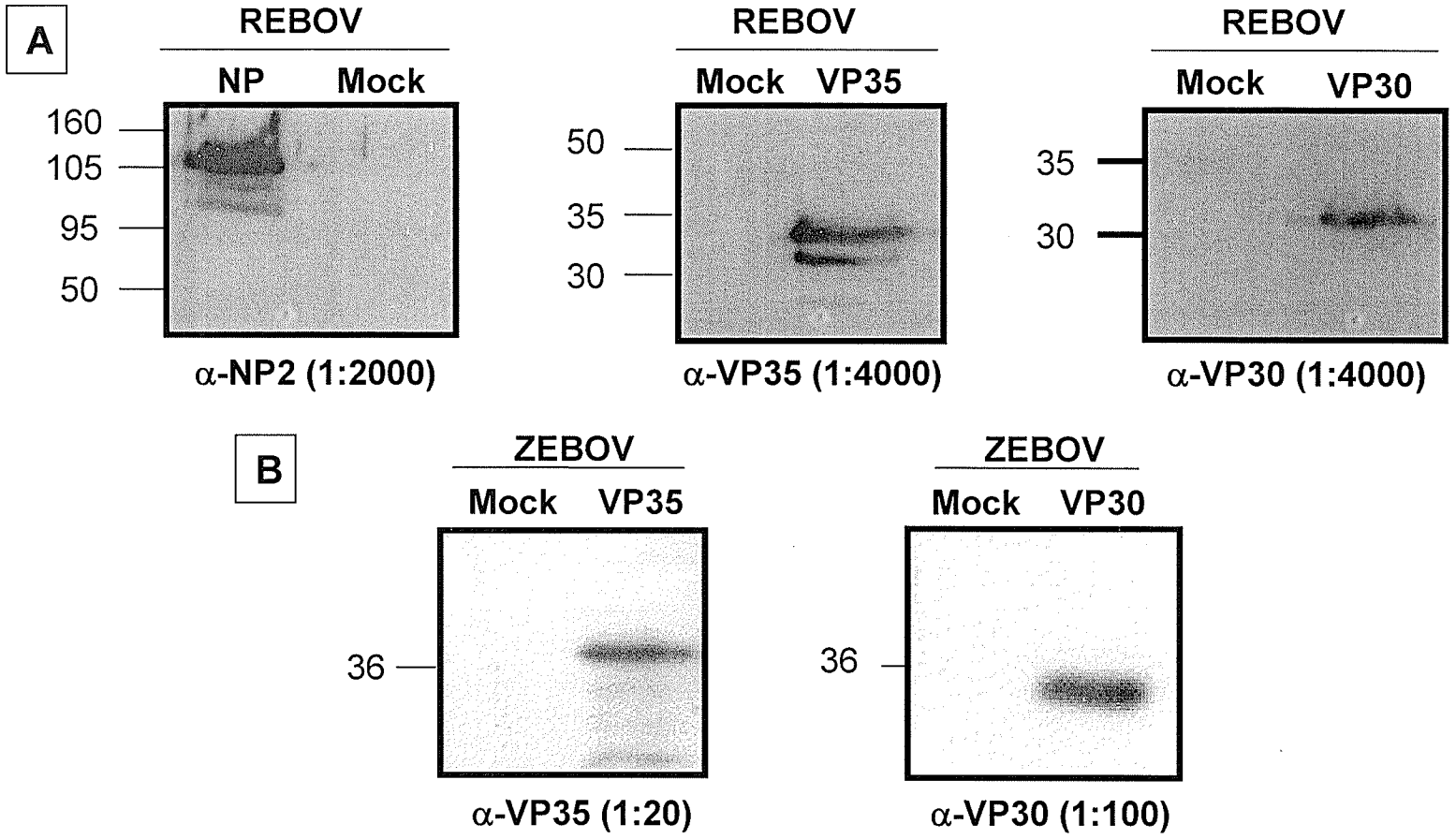



**Figure 21. Detection of RNP complex proteins from REBOV and ZEBOV by western blotting. (A) Detection of REBOV RNP complex proteins.** Cell lysates from 293T cells transfected with each of NP, VP35 or VP30 were separated by SDS-PAGE on 10% acrylamide gels and transferred to PVDF. Detection of protein expression was by western blot using antisera directed against REBOV NP2 peptide, REBOV VP35 and REBOV VP30, respectively. **(B) Detection of ZEBOV VP35 and VP30.** Cell lysates from 293T cells transfected with pCAGGS expression plasmids encoding either ZEBOV VP35 or ZEBOV VP30 were separated by SDS-PAGE in a 10% acrylamide gel, transferred to PVDF and probed by western blotting using REBOV antisera directed against VP35 or VP30, respectively.

represent degradation products of the full-length proteins or the products of internal ATG start codons.

## 3.3 Ribonucleoprotein Complex Protein Localization and Function

While the expression of RNP complex proteins from pCAGGS could be confirmed by antibody detection for three of the four components, NP, VP35 and VP30, it was still necessary to confirm expression of L by alternative means. In addition, the characteristics of these proteins had to be confirmed to be authentic and ultimately, the function assessed in an established system. One important characteristic for protein function is their ability to undergo specific protein-protein interactions, the formation of which have been closely tied to generation of a functional replicase complex (Becker et al., 1998).

### 3.3.1 Confirmation of Protein-Protein Interactions

It has been well documented for MARV that different components of the replicase complex are localized to different regions of the cell upon singular expression. (Becker et al., 1998). Further, interactions between specific MARV components leads to relocalization of these proteins into distinctive arrangements. In particular, NP of MARV is known to be localized in inclusion bodies and is capable of recruiting VP35 and VP30 out of their normal cytoplasmic distribution into these inclusions (Becker et al., 1998). Since the formation of NP-VP35 interactions is an important part of the replicase complex formation, with VP35 acting to bridge NP and L which cannot interact directly (Becker et al., 1998), and interaction of the EBOV replicase complex with VP30 is

critical for mRNA synthesis (Muhlberger et al., 1999, Weik et al., 2002), the ability of pCAGGS expressed proteins to undergo these interactions would be a reasonable indication of authentic structure and possibly also preservation of their functions. For REBOV, the pattern of protein-protein interactions would be predicated to be similar to that of MARV, based on both the conservation of protein function between the two filovirus genera and the apparent conservation of genomic structures related to regulation transcription and replication.

When singularly expressed it was observed that REBOV VP35 and VP30 were present diffusely throughout the cytoplasm, while for NP the formation of inclusion bodies was apparent (Figure 20B). Following cotransfection of either REBOV VP35 or VP30 together with NP, the formation of distinct inclusion bodies could be observed when staining was performed using an anti-VP35 or anti-VP30 primary antibody, respectively (Figure 22A). This indicated that both these components are capable of interacting with NP in a stable fashion, which leads to their recruitment into inclusions. While previous data using a flag-tagged form of MARV L expressed by coinfection with vaccinia virus expressing T7 indicated that this protein is expressed in the cytoplasm (Becker et al., 1998), coexpression of REBOV VP35 or VP30 together with an untagged REBOV L construct also showed relocalization of VP35 and VP30 into cytoplasmic inclusions in our assays (Figure 22A). This suggests that L may itself be localized in inclusion bodies, and thus able to mediate relocalization of VP35 and VP30 through its interactions with these proteins. Unfortunately the localization of L could not be directly confirmed due to the lack of antibodies to detect this protein.

Additionally, based on results using the ZEBOV infectious clone system, it was

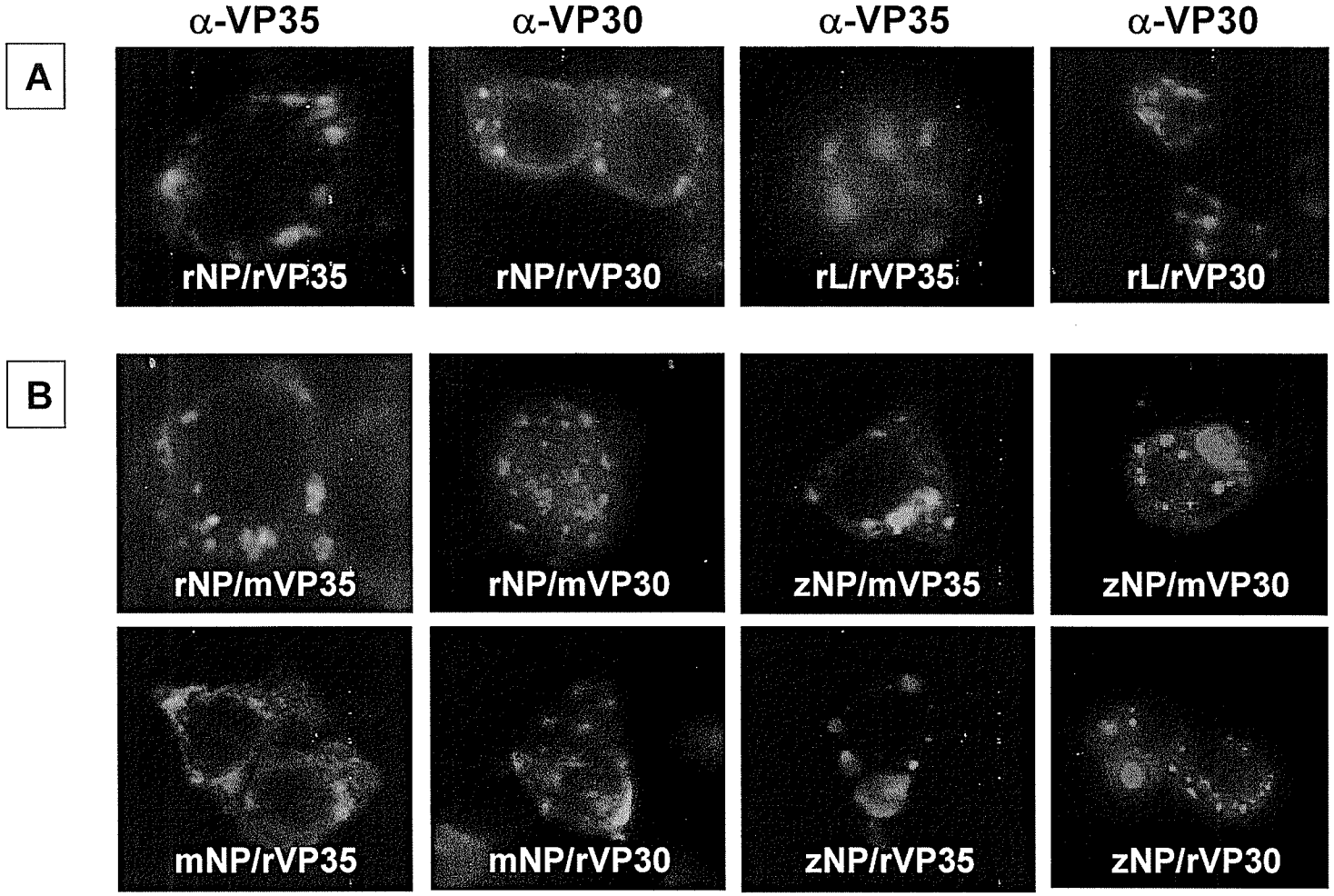



**Figure 22. Localization of RNP proteins following coexpression. (A) Coexpression of REBOV RNP components.** 293T ells were cotransfected with expression plasmids for NP+VP35, NP+VP30), L+VP35 or L+VP30 as indicated. Expression and localization of VP35 or VP30 in these combinations was detected with antisera directed against REBOV VP35 or VP30, respectively and compared to the localization patterns of these proteins when singularly expressed (as shown in Figure 20). **(B) Coexpression of heterologous RNP components.** Cotransfection into 293T cells of expression plasmids for NP from ZEBOV, REBOV or MARV together with VP35 or VP30 from either REBOV or MARV were visualized by IFA. Detection of combinations containing MARV VP35 or VP30 (top panel) were detected using a MARV anti-VP35 antiserum or MARV anti-VP30 monoclonal antibody, respectively. Detection of combinations containing REBOV VP35 or VP30 (lower panel) were detected using REBOV anti-VP35 or REBOV anti-VP30 antisera, respectively.

observed that individual components from a heterologous virus source could be introduced into the RNP complex of another filovirus and retain their function (Theriault et al., 2004) (Figure 23). However, one criticism of this work was the possibility that these interactions were only of a very transient nature and not biologically significant but an artifact of the replicative nature of infectious clone systems once the initial transcription event is achieved. In order to address this possibility we used relocalization by IFA to confirm that protein-protein interactions between RNP components from different sources were indeed occurring, since relocalization of proteins would require relatively stable interactions likely to be of biological significance. Based on the availability of specific antibodies to detect VP35 and VP30 from MARV and REBOV, NP from MARV, ZEBOV or REBOV was transfected together with VP35 and VP30 from MARV or REBOV. Following IFA using the anti-REBOV VP35 or anti-VP30 sera described above, an anti-MARV VP30 guinea pig serum or and anti-MARV VP35 mouse monoclonal antibody, relocalization of VP35 and VP30 into inclusion bodies could be observed in all cases, regardless of which virus species NP was derived from (Figure 22B). This suggests that protein-protein interaction between filovirus RNP components do occur in a stable fashion and that their ability to functionally complement each other, as seen in the ZEBOV infectious clone system Figure 23), is likely biologically relevant.

### 3.3.2 Confirmation of Function

Data obtained using the ZEBOV infectious clone system showed that rescue of virus from this system can be mediated by filovirus RNP complexes from a different virus source than the template (i.e. MARV RNP proteins could initiate transcription of a

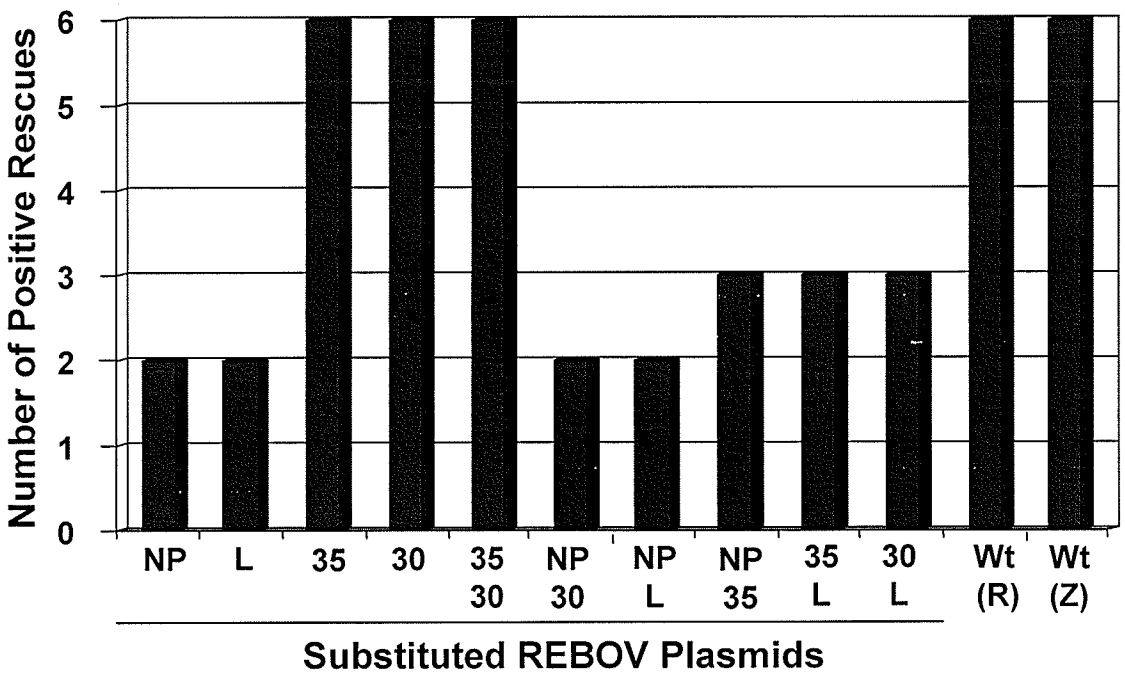



**Figure 23. Rescue of ZEBOV from the infectious clone system using REBOV RNP expression plasmid.** Individual REBOV (lanes 1-4) or pairwise combinations (lanes5-10) of REBOV RNP expression plasmids were substituted for their ZEBOV counterparts in the ZEBOV infectious clone system and their ability to mediate virus rescue determined. All expression plasmids for the REBOV (lane 11) or ZEBOV (lane 12) RNP complex were also transfected as part of the infectious clone system for comparison. The number of positive rescues out of 6 attempts with each plasmid combination was graphed (Modified from Theriault et al., 2004)

ZEBOV genome) (Theriault et al., 2004). This provided a unique opportunity to validate the function of the REBOV RNP complex protein expression plasmids prior to their use in establishing novel reverse genetics tools. Therefore, together with Steven Theriault, the ability of the REBOV RNP complex proteins to substitute for their equivalents in the ZEBOV infectious clone system was examined. It was found that when all four REBOV RNP proteins were supplied, ZEBOV could be efficiently rescued (Figure 23, lane 11). Alternatively, when single components were substituted into the ZEBOV RNP complex only VP35 and VP30 could mediate efficient rescue (Figure 23, lanes 1-4). In contrast, the rates of rescue for NP and L substitutions were only 33% (Figure 23, lane 1 and 2), indicating more stringent species specificity for these components. Similarly, pairwise substitutions involving NP and L showed lower rescue efficiencies than those involving only VP35 and VP30 (Figure 23, compared lane 5 and lanes 6-10). Together with their implications for species-specificity of RNP complex protein interactions, these data were sufficient to demonstrate that each of the REBOV RNP complex proteins was being expressed in a form which was functional to mediate viral transcription of a closely related viral genome and thus suitable for use in establishing new REBOV-based reverse genetics tools.

## 3.4 REBOV Minigenome System Development

With adequate confirmation that all of the RNP complex components for REBOV could be expressed in a functional form, the next step in the development of reverse genetics for REBOV was to establish a monocistronic minigenome system. In addition to the RNP complex proteins, this required the construction of minigenome plasmids.

Based on the availability of good quality sequencing data for the genomic ends, as a result of our full-length genome sequencing work, we were able to construct such plasmids.

### 3.4.1 Rescue of Reporter Activity from a Pol I-Driven Minigenome

In order to establish a minigenome system for REBOV, monocistronic minigenomes consisting of the following elements: 3' non-coding region (leader) – NP untranslated region – reporter- L untranslated region - 5' non-coding region (trailer) were constructed. Constructs were generated which contained either chloramphenicol acetyl transferase (CAT) or enhanced green fluorescent protein (GFP) as the reporter and were oriented such that transcription generated a vRNA-like molecule (vRNA-oriented construct) (Figure 8). Both the CAT and GFP-containing reporter cassettes were cloned under the control of a human RNA Pol I promoter in pRF240 (Flick et al., 2003a, Flick et al., 2003b). In order to evaluate the function of these minigenome constructs prior to the generation of the necessary RNP expression helper plasmids required to drive transcription and replication entirely from plasmid DNA, helpervirus infection with REBOV was used to mediate reporter gene expression. For this experiment, varying amounts of the Pol I- CAT minigenome plasmid were transfected into 293T cells, followed by infection with REBOV 24 h later using multiplicities of infection (MOIs) of 0, 0.1 or 0.01. Samples were harvested at different time points post infection and analyzed for CAT activity. Figure 24A shows the data obtained 72 h post infection. At this time point the best signal-to noise ratios were obtained using 0.25 μg of transfected minigenome and an MOI of 0.001. The decrease in reporter rescue observed with a

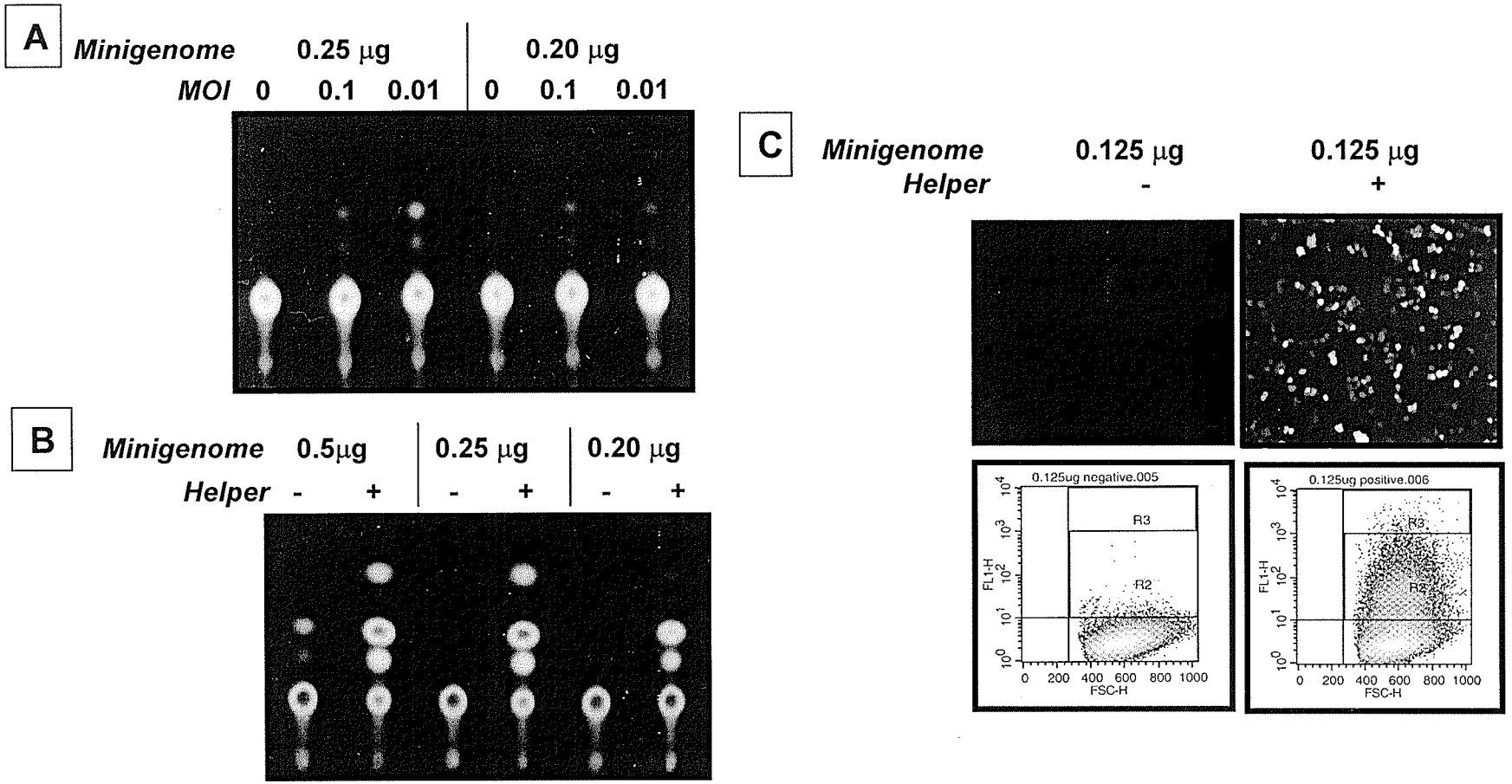



**Figure 24. Pol-I-driven REBOV minigenome transcription (A) Infection-driven Pol I-CAT minigenome transcription.** Decreasing amounts of Pol I-driven vRNA-oriented CAT minigenome were transfected into 293T cells and and incubated for 24 h prior to REBOV infection with an MOI of 0, 0.1 or 0.01. Cells were incubated for a further 72 h at 37°C with 5% $CO_2$ prior to lysis and assay for CAT activity. CAT activity was determined by incubating 20µl of lysate overnight in the presence of chloramphenicol and acetyl coenzyme A. **(B) Helper plasmid-driven Pol I-CAT minigenome transcription.** Decreasing amounts of Pol I-driven vRNA-oriented CAT minigenome were transfected as described in (A) along with standard amounts of helper plasmid (1.0ug NP, 0.5ug VP35, 0.3ug VP30 and 1.0ug L) into 293T cells and incubated for 48 h. Cells were then lysed and assayed for CAT activity by incubation of 5µl cell lysate for 2 h in the presence of chloramphenicol and acetyl coenzyme A. **(C) Helper plasmid-driven Pol I-GFP minigenome transcription shown by UV fluorescence.** Samples were transfected as described in (B) and analyzed after 24 h. Data are shown for the transfection of 0.125ug of minigenome, which yielded optimal results when data were analyzed by UV microscopy of FACS analysis. (Modified from Groseth et al., 2005)

higher MOI may be related to virus-induced changes in host cell functions that effect protein translation, or competition between the minigenome and full-length genome, perhaps for interaction with a cofactor whose availability is limiting. Nevertheless, helpervirus 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, it was necessary to establish a set of conditions which would allow reporter transcription in a plasmid-driven system. For this, we began by transfecting various amounts of the Pol I-CAT minigenome construct in the presence or absence of the necessary helper plasmids (NP, VP35, VP30 and L). The amount of each of the helper plasmids supplied was based on the optimal values previously determined for the T7-driven ZEBOV minigenome and infectious clone systems (Muhlberger et al., 1999, Neumann et al., 2002) and was as follows: NP – 1.0 μg, VP35 – 0.5 μg, VP30 – 0.3 μg, L – 1.0 μg.

Based on these input minigenome titration experiments, it was found that transfection of 0.25 μg of vRNA-oriented Pol I-driven CAT minigenome was sufficient to produce CAT activity nearly equal to that obtained with higher amounts of reporter (compare 0.5 μg of transfected minigenome) in the presence of the required helper plasmids (Figure 24B). Importantly, there was no detectable background reporter activity in the absence of helper plasmids using this amount of transfected minigenome (Figure 24 B). The background activity, observed in the presence of higher amounts of reporter minigenome, likely results from the recognition of cryptic promoter elements contained within the minigenome plasmid by host polymerases (Flick et al., 2003a, Flick &

Pettersson, 2001). 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 non-coding regions by the viral RNP complex proteins, thus, allowing high level of reporter activity in the absence of appreciable background. As shown with minigenome systems for other viruses previously (Flick & Pettersson, 2001), the plasmid-driven REBOV Pol I minigenome system provided superior reporter expression compared to the helpervirus-driven one (Figure 24A versus Figure 24B).

In order to determine if these data could be replicated using an alternative reporter, the same protocol as was used for the helper plasmid-driven CAT minigenome experiment was followed using a Pol I-driven GFP construct, except that GFP expression was monitored by UV microscopy and FACS analysis (Figure 24C). It was determined for this construct that even 0.125 μg of input minigenome still provided a very high signal intensity in a large proportion of the cell population in the absence of appreciable background. These data indicate that the results obtained with this system are reproducible using different reporter protein activities as an indication of minigenome transcription.

### 3.4.2 Optimization of Helper Plasmid Concentrations

While initial helper plasmid-driven minigenome experiments were carried out using helper plasmid masses based on the optimal values previously determined using the T7-driven ZEBOV minigenome and infectious clone systems (Muhlberger et al., 1999, Neumann et al., 2002), it was unclear whether the same input masses would be ideal for REBOV. This was particularly of concern since transcription/replication in a MARV

system has been reported to have substantially different protein input optima than were reported for a similar ZEBOV system (Muhlberger et al., 1998, Muhlberger et al., 1999). Therefore, 0.25 μg of Pol-driven CAT minigenome was transfected into 293T cells along with various amounts of helper plasmids in order to determine what effect this would have on reporter output. Specifically, the transfected mass of each protein was increased 2x and 4x, as well as decreased to ½ and ¼ of the levels reported to be optimal in the ZEBOV systems. Using this approach we were able to confirm the optimal values for ZEBOV also appear to be reasonable optima for the transcription of REBOV minigenomes (Figure 25), an observation that further emphasized the similarities in the transcription and replication strategies employed by different EBOV species. The one exception from this was the observation that reporter output was increased with increasing input levels of L up to 4x that starting amount (Figure 25), although this observation has since been confirmed in the ZEBOV minigenome system (Watanabe et al., 2004). However, the resulting increases in reporter activity obtained were relatively modest (~20%) (Figure 25) and, therefore, we did not deem the benefits of this modification to be worth the substantial increase in plasmid consumption. Thus, the protocol was continued using 1.0 μg of NP, 0.5 μg of VP35, 0.3 μg of VP30 and 1.0 μg of L.

### 3.4.3 Confirmation of Replication

In the initial experiments all reporter cassettes were cloned in vRNA orientation, meaning that the reporter gene was in anti-sense orientation on the primary Pol I or T7 transcript (Figure 8). In order to confirm that replication, in addition to the viral

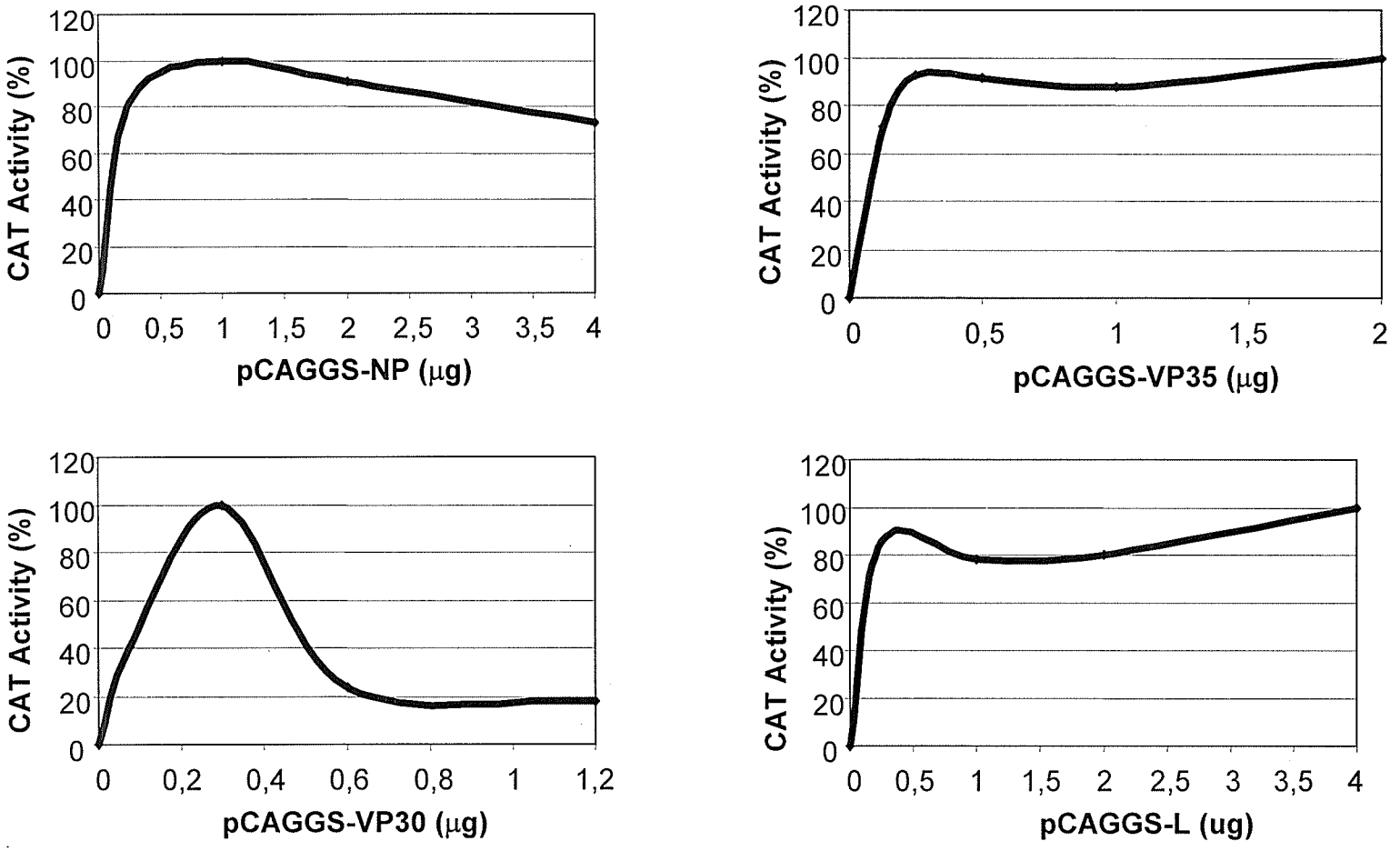



**Figure 25. Optimization of input helper plasmid.** Various amounts of helper plasmid were transfected along with 250 ng of Pol I-driven vRNA-oriented CAT minigenome into 293T cells and incubated for 24 h at 37°C with 5% $CO_2$. The cells were incubated for a further 48 h at 37°C with 5% $CO_2$ prior 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. (Modified from Groseth et al., 2005)

transcription shown by the methods described above, was occurring, the CAT reporter cassette was also cloned for Pol I-driven transcription in cRNA orientation. This construct resulted in a Pol I reporter gene transcript in which the reporter gene is in sense orientation and would, therefore, have to first be replicated to form a vRNA-like molecule in order to be functional for transcription (Figure 8). Thus, 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 using the T7-driven ZEBOV infectious clone system, showed enhanced virus rescue from a cRNA-oriented genome (Neumann et al., 2002) and we were, therefore, interested to determine whether enhanced reporter activity could be obtained using a cRNA-oriented minigenome construct. For these experiments 293T cells were transfected with varying amounts of cRNA-oriented minigenome and established concentrations of helper plasmids. However, CAT activities, measured after 48 hours (Figure 26A), were universally lower compared to the vRNA-oriented constructs (Figure 24B) when equivalent masses of minigenome and helper plasmids were used for transfections.

It is likely that these apparently conflicting findings derive from differences in both the read-out and the biology of these systems. In addition to the possibility of dsRNA formation in the infectious clone system, due to hybridization of the RNP plasmid mRNAs and the vRNA-oriented viral genome, the need to produce infectious particles required the generation of large number of progeny vRNA copies, a function which is in turn dependant on the production of the cRNA intermediate. The ready availability of these cRNA intermediates in this system may, therefore, ensure sufficient

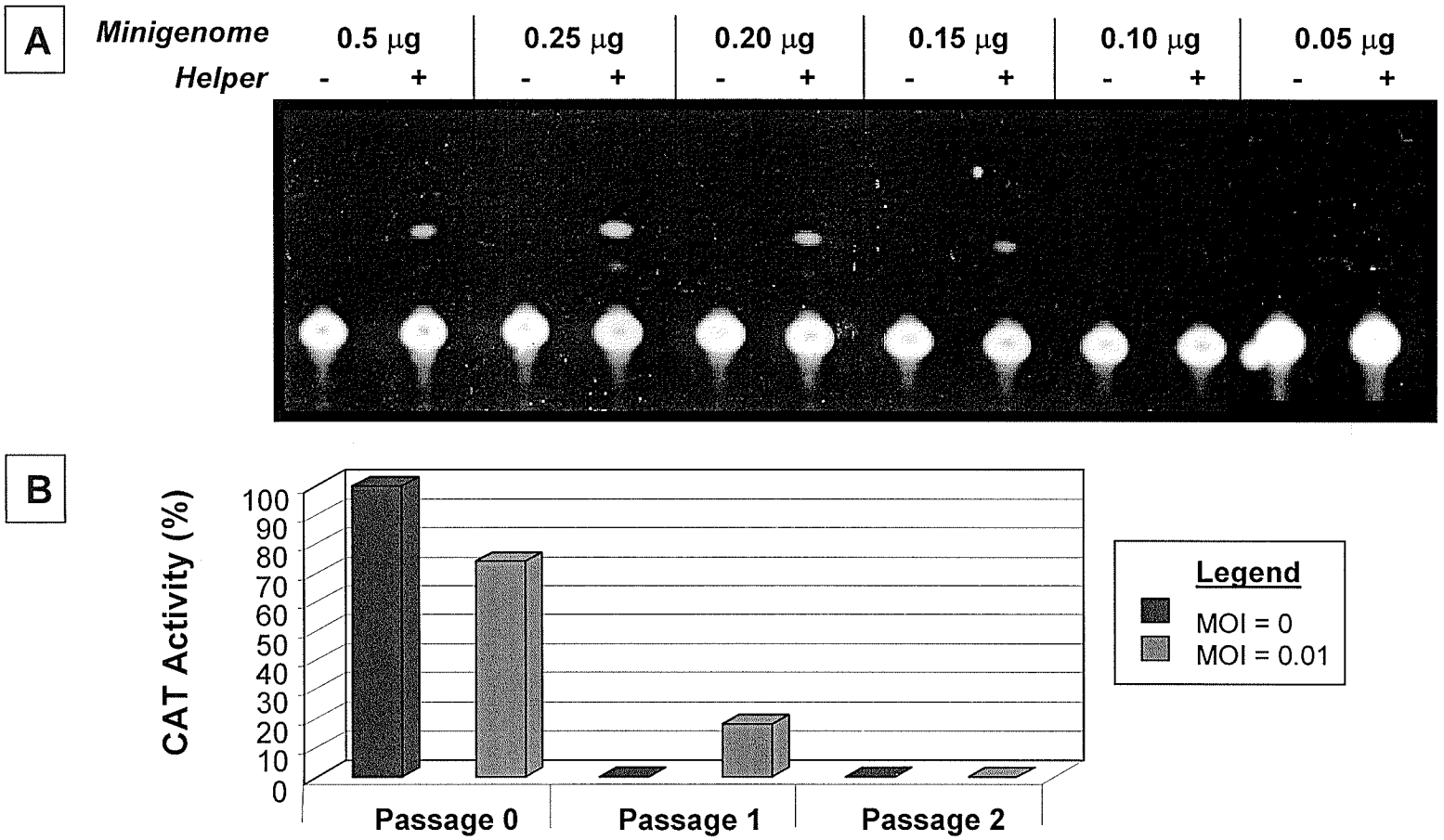



**Figure 26. Replication and Packaging of REBOV Pol-I minigenomes (A) cRNA-oriented Pol I driven minigenome replication.** Various amounts of a Pol I-driven cRNA-oriented CAT minigenome were co-transfected along with standard amounts of each helper plasmid (1.0ug NP, 0.5ug VP35, 0.3ug VP30 and 1.0ug L) into 293T cells. Cultures were incubated for 48 h prior to the cell lysates being harvested. In order to determine CAT activity 5µl of cell lysates was incubated for 2 h in the presence of chloramphenicol and acetyl coenzyme A and compared to the results obtained using VRNA-oriented constructs (as shown in Figure 24). **(B) Minigenome packaging into virions.** Following transfection of 0.25ug of vRNA-oriented Pol I-driven CAT minigenome and standard amounts of helper plasmid (1.0ug NP, 0.5ug VP35, 0.3ug VP30 and 1.0ug 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 further incubation for 72 h, cell lysates were harvested and 2 ml of undiluted supernatant was transferred to fresh monolayers. This procedure was repeated twice more with CAT activity of cell lysates being determined after each passage. (Modified from Groseth et al., 2005)

vRNA production, in relation to protein levels, for enhanced virus production. In contrast, in the minigenome system there are no potential targets for helper plasmid mRNA hybridization on the vRNA and the read out is based strictly on viral transcription of reporter mRNA. If the transcriptional output of the Pol I promoter is superior to that of the viral replicase then this system would favour direct production of vRNA rather than its replication though a cRNA intermediate.

### 3.4.4 Confirmation of Packaging

The final element of the successful establishment of a minigenome system is the demonstration of packaging of minigenome transcripts into virus particles through recognition of the minimal packaging signals in the 3' and 5' non-coding regions. This can be demonstrated by the ability of reporter activity to be passaged from an initial culture to fresh cell monolayers. 293T cells were, therefore, initially transfected with both the Pol I-driven CAT minigenome and the necessary helper plasmids. This resulted in efficient transcription of vRNA-like reporter molecules as indicated by high levels of CAT activity (Figure 26B). In the absence of an infecting helpervirus 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 compared to those that were infected (Figure 26B). However, REBOV infection 24 hours after transfection of the minigenome plasmid and RNP complex protein encoding plasmids was able to transfer reporter activity to fresh cell cultures. Following the first passage, 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, packaging

of minigenome vRNA occurred indicating the presence of the minimal necessary *cis*-acting signals for packaging within the non-coding genome regions flanking the reporter gene.

While the efficiency observed for REBOV packaging in this system was relatively low it was consistent with that reported for previously published systems (Flick et al., 2004, Flick & Pettersson, 2001) and may be a result of the poor growth of REBOV in tissue culture, compared to other filoviruses. In particular, if virus titres sufficiently high to mediate superinfection of target cells were not achieved before supernatant transfer, both a minigenome and a full-length genome (to supply additional RNP components) would need to be incorporated in a single particle. This packaging of multiple RNPs would likely be complicated by the highly ordered structure of filovirus particles.

### 3.4.5 Comparison to a T7-driven Minigenome System

Encouraged by the potency of the Pol I-driven minigenome system, we wished to compare the efficiency of this novel REBOV Pol I transcription system to one based on T7-driven transcription, since this is clearly the standard for reverse genetics for filoviruses (Boehmann et al., 2005, Hoenen et al., 2006, Muhlberger et al., 1998, Muhlberger et al., 1999, Neumann et al., 2002, Volchkov et al., 2001, Watanabe et al., 2004). In order to assess this, the CAT reporter cassette was cloned in vRNA orientation under the control of the T7 promoter in a specially constructed derivative of pBSK(-) containing the hepatitis delta virus (HDV) ribozyme and T7 terminator sequences in the appropriate arrangement. In addition, a pCAGGS-T7 expression plasmid was obtained

for use as a source of T7 polymerase (kindly provided by Dr. Yoshihiro Kawaoka, University of Tokyo). We then determined reporter expression from both the T7-driven and Pol I-driven CAT minigenomes, in the presence or absence of the necessary helper plasmids. Surprisingly, T7-driven REBOV minigenomes produced only weak CAT signals, when transfected in amounts found to produce very strong CAT reporter signals with the Pol I-driven system (Figure 27, top panel vs. middle panel). These signals could be enhanced by increasing the incubation time of the CAT reaction from 2 h to 18 h (Figure 27, lower panel), but even at this time point the levels of reporter activity did not reach those obtained by the Pol I system after only 2 hours. In addition, following the prolonged incubation required to obtain strong reporter signals with the T7-driven REBOV minigenome, substantial levels of background became apparent, while background reporter expression is not normally observed using the Pol I-driven minigenome system with less than 0.5 μg of minigenome (Figure. 24B). Thus, in the case of REBOV, the Pol I-driven minigenome system seems much more potent than the T7-driven system.

### 3.4.6 Effect of Purine Residues on T7-driven Minigenome Transcription

One potential point of consideration for the T7-driven system that was investigated in order to maximize reporter expression from this construct was the effect of G residues in the +1 to +3 positions on the efficiency of transcription by T7. The minimal T7 promoter sequence is defined as $^{-17}$-TAATACGACTCACTATA**G**GG$^{+3}$ , with the +1 residue shown in bold (Milligan et al., 1987). While the +3 to +6 residues are

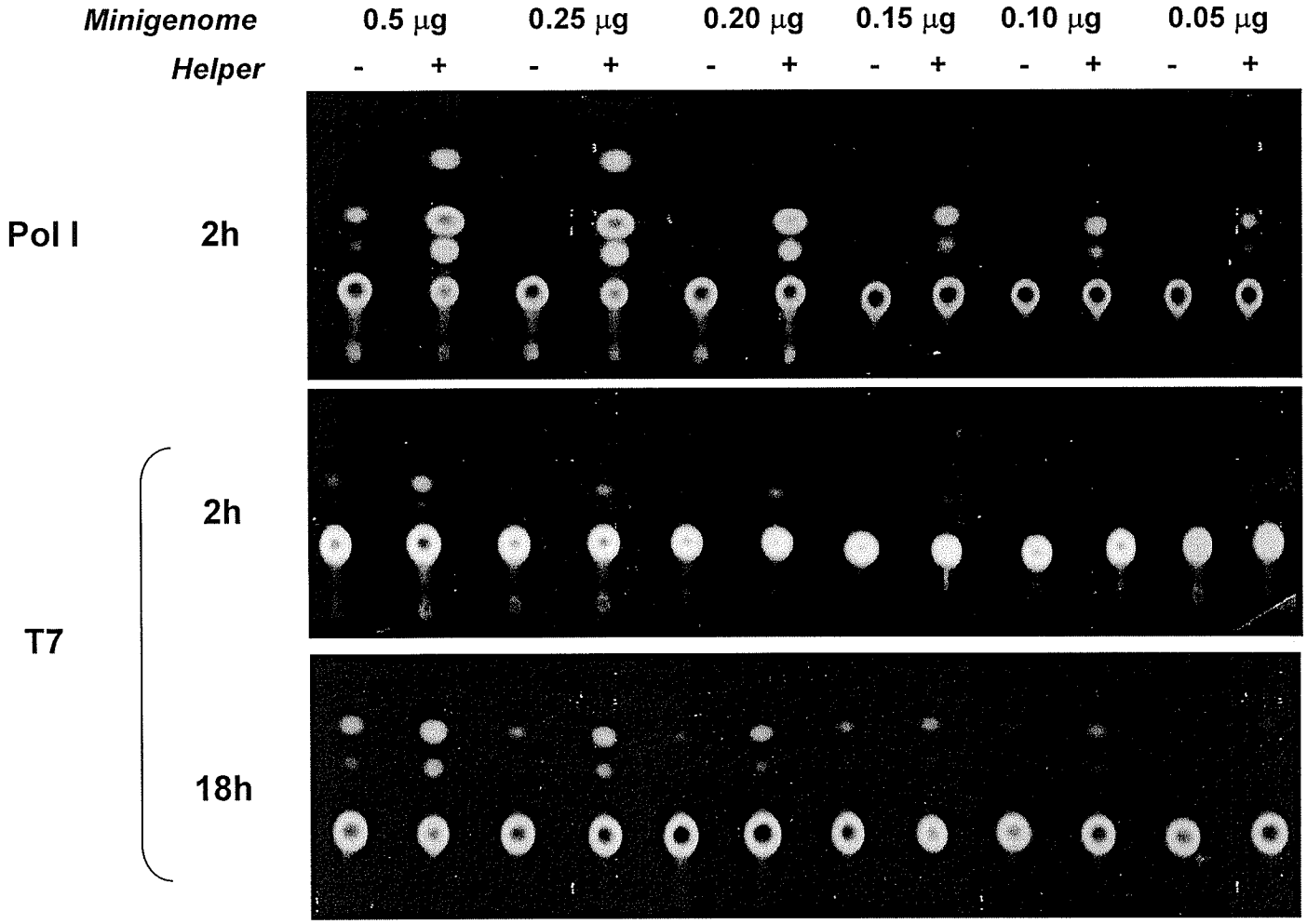



**Figure 27. Comparison of Pol I- and T7- mediated transcription of CAT reporter minigenomes.** Varying amounts of T7 or Pol I-driven CAT minigenome was transfected in addition to standard amounts of helper plasmids (1.0ug NP, 0.5ug VP35, 0.3ug VP30 and 1.0ug L). In the T7-driven system, 1.0ug of a pCAGGS construct expressing the T7 polymerase was also transfected. The relative amounts of reporter expression produced by the two systems were compared following incubation of 5μl of cell lysate in the presence of chloramphenicol and acetyl coenzyme A for 2 h. In order to obtain more comparable signals, the T7-driven minigenome lysates were also incubated for 18 h. (Modified from Groseth et al., 2005)

relatively flexible (Milligan et al., 1987), and therefore, often altered to coincide with the gene sequence to be transcribed, alteration of the G residues present in the +1 or +2 sites can significantly decrease the efficiency of transcription from this promoter sequence (Milligan et al., 1987). However, many viral genomes, including those of filoviruses, are believed to form panhandle structures between the 3' and 5' termini or, as indicated by more recent evidence for filoviruses, terminal stem-loop structures (Weik et al., 2005). The formation of either of these structures would be dependent on base-pairing interactions and are likely important for transcription and replication by the viral polymerase. It is not know what effect the additional non-coded G residues incorporated as part of the T7 promoter might have on the formation and/or recognition of regulatory elements in these regions. To date, reverse genetics tools for filoviruses have incorporated a single G residues in the +1 position as a compromise between maximal transcription from the T7 promoter and preservation of the genome end sequences (Muhlberger et al., 1998, Muhlberger et al., 1999, Neumann et al., 2002, Volchkov et al., 2001). However, no data validating this approach to filovirus reverse genetics construction has yet been published. Thus, it was of interest to us to determine if incorporation of G residues in the +1, +1/+2, +1/+2/+3 positions of the T7 promoter sequence would have an impact on reporter activity in the minigenome system. In this case, reporter production should reflect the net effect of increased T7 transcription as well as any potentially deleterious effects on genome end interactions and subsequent recognition by the RNP proteins. Accordingly, T7-driven minigenome constructs were generated by site-directed mutatgenesis that contained 0, 1, 2 or 3 G residues upstream of the minigenome cassette (Figure 28A). However, analysis of these constructs

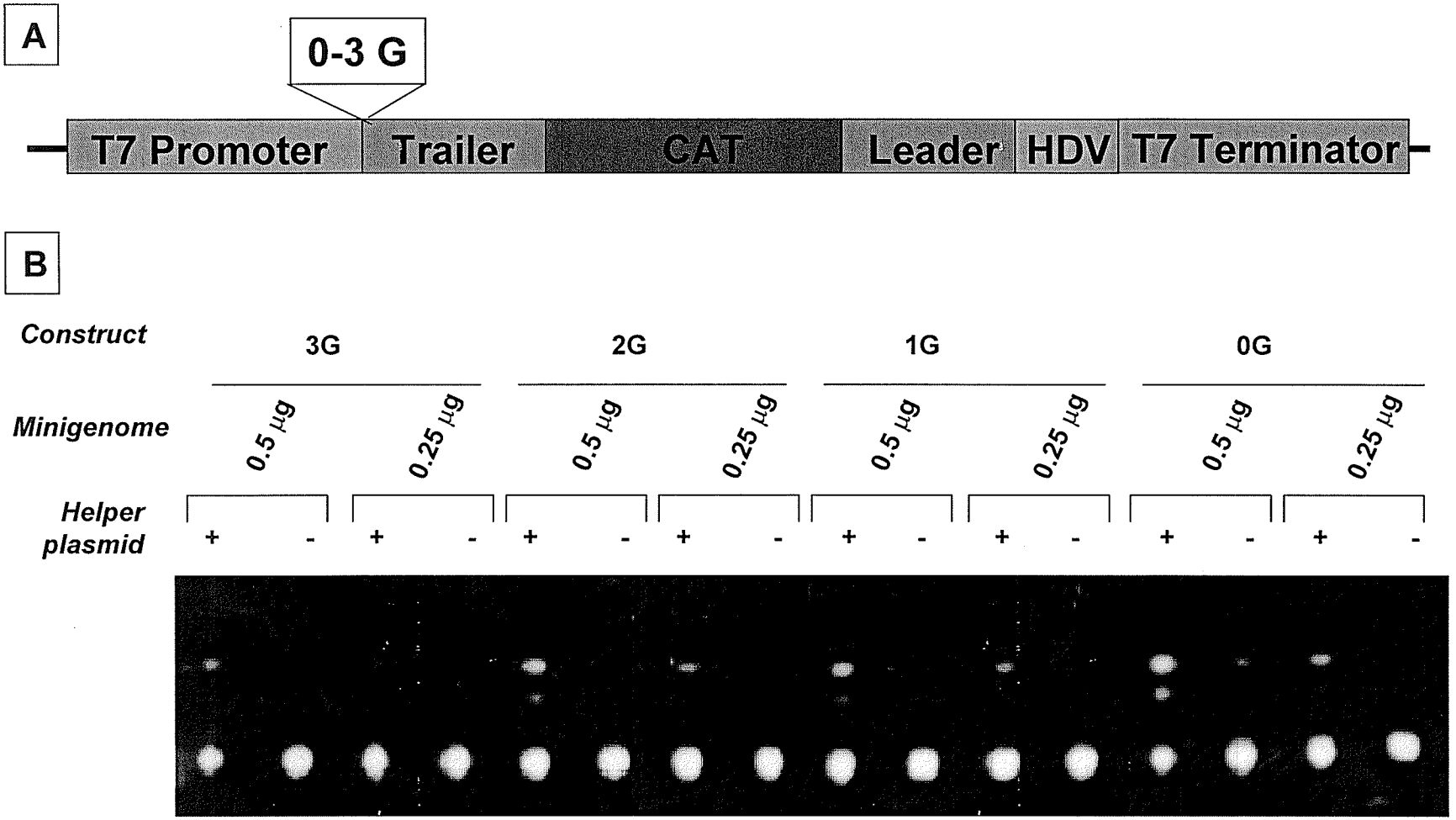



**Figure 28. Effect of upstream G residues on transcription of T7-driven minigenome constructs. (A) Structure of upstream G containing T7-driven minigenome plasmids.** Through site-directed mutagenesis 1, 2 or 3 G residues were added to an existing O G T7-driven minigenome construct. **(B) Transcription of G residue containing T7-driven minigenomes.** Each plasmid was transfected in 0.5 µg and 0.25 µg amounts, in addition to standard amounts of helper plasmids (1.0ug NP, 0.5ug VP35, 0.3ug VP30 and 1.0ug L) and 1.0ug of a pCAGGS construct expressing the T7 polymerase. The resulting reporter activity measured as an indication of the level of comparative T7 transcription occurring.

demonstrated very little effect on reporter activity as a result of incorporation of these additional G residues (Figure 28B). If at all, reporter activity was marginally higher in experiments performed using the T7 construct lacking all of the additional G residues (Figure 28B), indicating that preservation of genome end interactions is more important than enhancing the efficiency of the initial transcript production in the REBOV minigenome system.

### 3.4.7 Comparison of Minigenome Transcription by Heterologous Ribonucleoprotein Complexes

As mentioned previously, one of the long-term goals of developing reverse genetics tools for REBOV is to study differences in the pathogenicity of filoviruses and to identify molecular determinants associated with these differences. One notable difference between REBOV and ZEBOV, besides the reduced virulence demonstrated by REBOV, is that REBOV displays significantly slower growth in tissue culture (i.e. 14 days for growth to $10^6$ ffu/ml from an MOI of 0.01, as compared to 7 days for similar growth of ZEBOV). We were interested to assess whether significant differences existed between the rates of transcription by various filovirus replicases, an aspect of virus biology that could be conveniently studied using the minigenome system. In particular, the ability to interchange RNP components on a single minigenome template would allow us to rule out differences in initial minigenome transcription levels as well as any genome specific influences.

While previous attempts to detect reporter gene expression from MARV or ZEBOV minigenomes using heterologous helper plasmids have failed (Muhlberger et al.,

1999), the success reported by Theriault *et al.* using the ZEBOV infectious clone system (Theriault et al., 2004) led us to revisit this issue using newly developed RNA Pol I minigenome system. In addition to the Pol-I REBOV minigenome system, a T7-driven MARV minigenome plasmid, which was developed by others in our lab and kindly provided by S. Theriault and G. Mehmetoglu, was also used. RNP complex expression plasmids for ZEBOV and MARV were provided through collaboration with the Philipps Universität Marburg (kindly provided by S. Becker) or constructed in our lab (kindly provided by S. Theriault and G. Mehmetoglu), respectively. For these experiments 293T cells were co-transfected with the optimized concentrations of the Pol I-driven REBOV minigenome plasmid (0.25 μg) and the 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). Cells were harvested and CAT activity was measured 48 h later. Similarly, 2.0 μg of MARV minigenome was transfected along with 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) and cells harvested and assayed for CAT activity after 48 h. Unexpectedly, transcription occurred at a similar level using either the MARV or ZEBOV RNP plasmids, on either template, regardless of the phylogenetic relationship between minigenome template and RNP complex (Figure 29A and B). These levels were also significantly higher than those obtained using the REBOV proteins for either the REBOV or MARV template (Figure 29A and B). These data not only clearly indicated the ability of the RNP complex to recognize the appropriate signals on heterologous minigenome RNAs, but also suggest that there are fundamental differences in the efficiency of transcription mediated by the viral replicases of virulent and avirulent filovirus species.

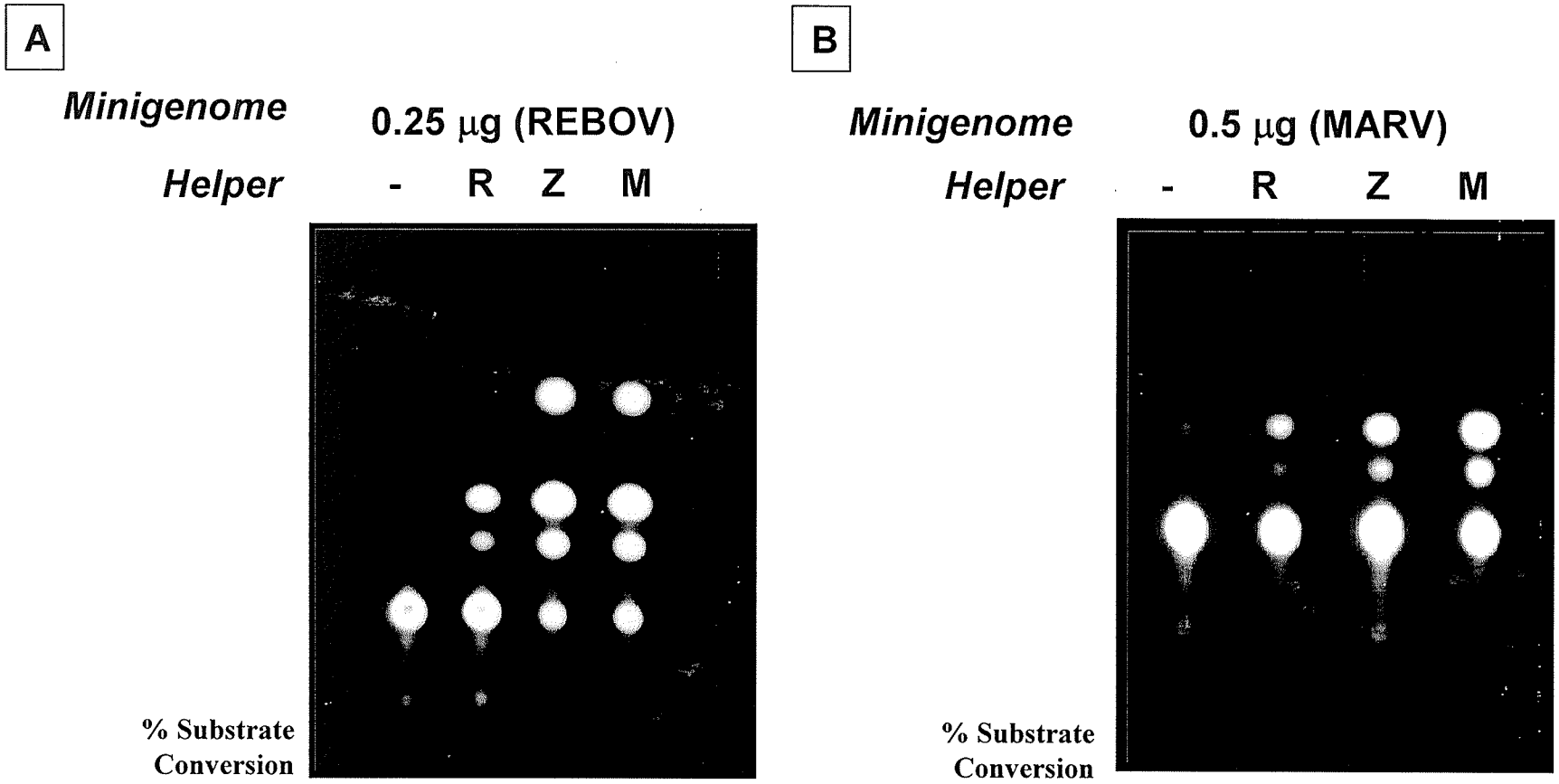



**Figure 29. Comparison of minigenome transcription by ribonucleoprotein complex components from *heterologous sources*. (A) REBOV minigenome system**. 0.25ug of vRNA-oriented Pol I-driven minigenome was co-transfected along with standard amounts of helper plasmids (1.0ug NP, 0.5ug VP35, 0.3ug VP30 and 1.0ug L) derived from either REBOV, ZEBOV or MARV. Transfected 293T cultures were then incubated for 48 h and assayed for CAT activity by incubation of 5μl of cell lysate in the presence of chloramphenicol and acetyl coenzyme A for 2 h. Substrate conversion was measured using the Typhoon phosphoimager. **(B) MARV minigenome system.** 0.5 ug of vRNA-oriented T7-driven minigenome was co-transfected along with standard amounts of helper plasmids derived from either REBOV, ZEBOV or MARV and 1ug of PCAGGS-T7. Transfected 293T cultures were then incubated for 48 h at and assayed for CAT activity by incubation of 5μl of cell lysate in the presence of chloramphenicol and acetyl coenzyme A for 2 h. (Modified from Groseth et al., 2005)

## 3.5 Regulation of Transcription by VP30

In a further attempt to apply our newly developed REBOV minigenome system to the study of filovirus transcription and replication, we were interested to investigate the basis for regulation of EBOV, but not MARV transcription, by VP30 (Muhlberger et al., 1999). In the ZEBOV minigenome system VP30 has been clearly shown to be involved in the regulation of EBOV transcription by acting as an anti-termination factor for synthesis of the NP mRNA (Weik et al., 2002). This process was shown to be dependant on the formation of a hairpin structure upstream of the NP open reading frame (Weik et al., 2002). However, it has never been adequately addressed why MARV NP transcription occurs independent of VP30. This issue has been further confounded by the observation that rescue of the infectious clone system for MARV is dependent on the transfection of VP30, but does not require an active Cys(3)-His Zn-binding domain (Enterlein et al., 2006), which has previously been shown to be essential for transcriptional activation by VP30 in EBOV (Modrof et al., 2003).

### 3.5.1 Confirmation of Filovirus Regulation by VP30

As a starting point for subsequent investigations into filovirus transcription by VP30 the effect of omission of VP30 or L on minigenome transcription was assessed in the Pol I-driven CAT REBOV minigenome system, the T7-driven CAT MARV minigenome and a T7-driven Luciferase (Luc) ZEBOV minigenome system, which was kindly provided through collaboration with S. Becker and T. Hoenen (Philipps Universität Marburg). Using these systems we could show that VP30 is required for REBOV transcription (Figure 30A), consistent with the idea that VP30 is an

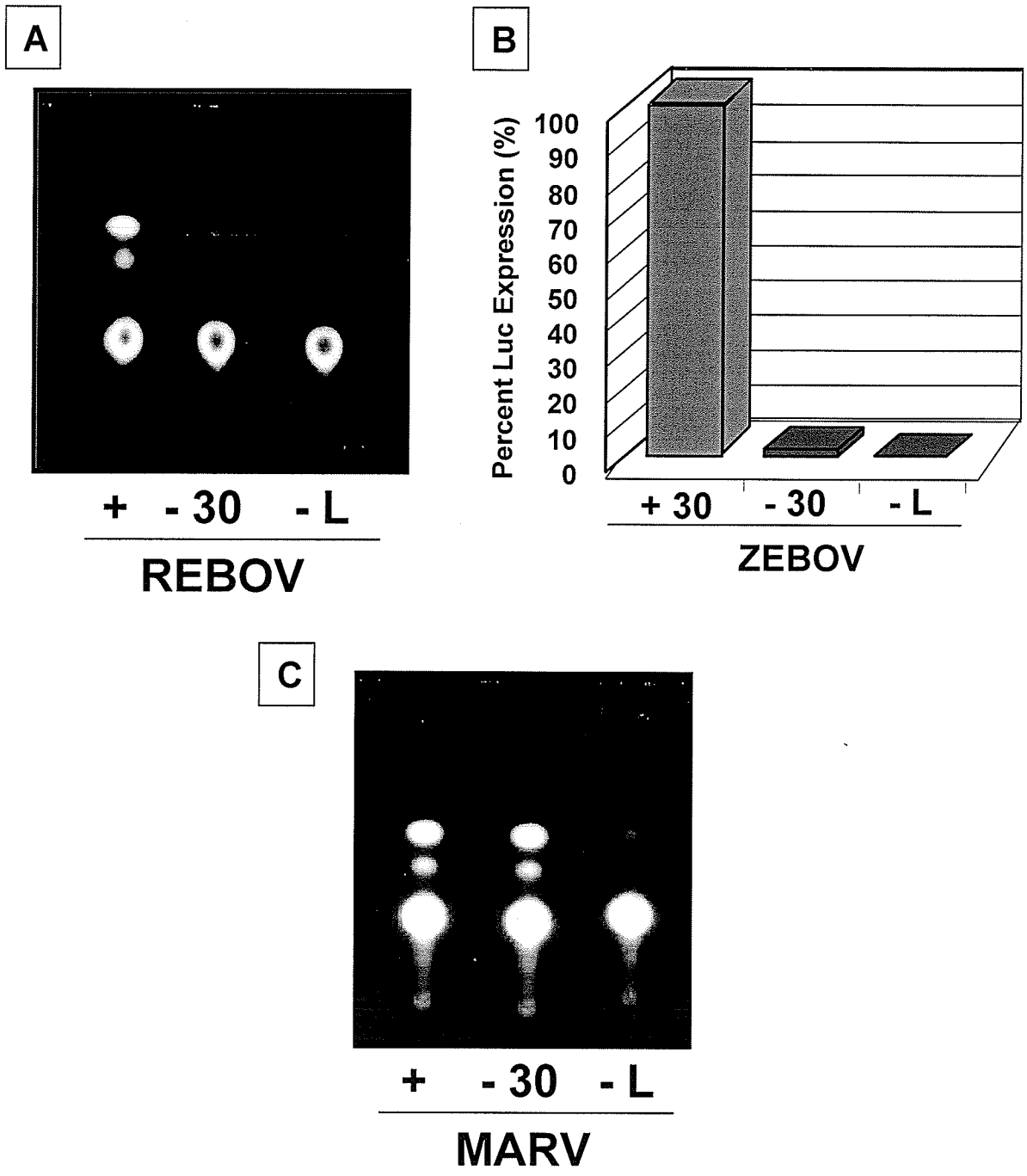



**Figure 30. Regulation of filovirus minigenome transcription by VP30. (A) REBOV minigenome.** The Pol I REBOV CAT minigenome, along with all REBOV RNP components or in the absence of either VP30 or L, was transfected in 293 T cells. Samples were incubated for 48h prior to harvest and assay for CAT activity. **(B) ZEBOV minigenome.** Transfection was carried out as described in (A) using the T7 ZEBOV Luc minigenome and ZEBOV RNP components. In addition, 1ug of pCAGGS-T7 was transfected. Samples were analyzed by luciferase assay after 48 h. **(C) MARV minigenome.** Transfection was carried out as described in (A) using the T7 MARV CAT minigenome along with MARV RNP components and 1ug of pCAGGS-T7. Samples were incubated for 48h prior to harvest and assay for CAT activity.

EBOV-specific transcription factor. Further, we could confirm that, as previously reported (Muhlberger et al., 1998, Muhlberger et al., 1999) transcription of ZEBOV was dependent on the presence of VP30, while transcription of MARV was not (Figure 30B and C). In all cases the omission of the viral RNA polymerase (L) resulted in a loss of reporter activity (Figure 30A, B and C), as expected.

### 3.5.2 RNA Folding Predictions for Genome Termini

Having set up the transcription systems required to address the question of a basis for genus-specific regulation of transcription by VP30, the possibility that these differences arise from differences in genomic structure was considered. Since regulation of NP transcription by VP30 in EBOV is related to the presence of a hairpin structure upstream of this gene, it appeared likely that the basis for this difference derived from the absence of such a hairpin in the MARV genome, and also the minigenome. In order to assess this possibility, structural predications for folding patterns in the minigenome sequence were generated using the M-fold tool for prediction of RNA secondary structure by free energy minimization. (Zucker, 1989). Based on these predictions it was observed that, similar to ZEBOV and REBOV, MARV may contain a hairpin structure upstream of the NP open reading frame (Figure 31). Further, this hairpin is predicted to be similar to those of the Ebola viruses in the fact that it overlaps the transcriptional start site for NP (Figure 31). One interesting difference between the hairpins, however, is the length of the hairpins. Indeed the MARV hairpin is predicted to extend an additional 8 bp longer than seen in EBOV before forming its loop region (Figure 31), although it is unclear what impact this might have.

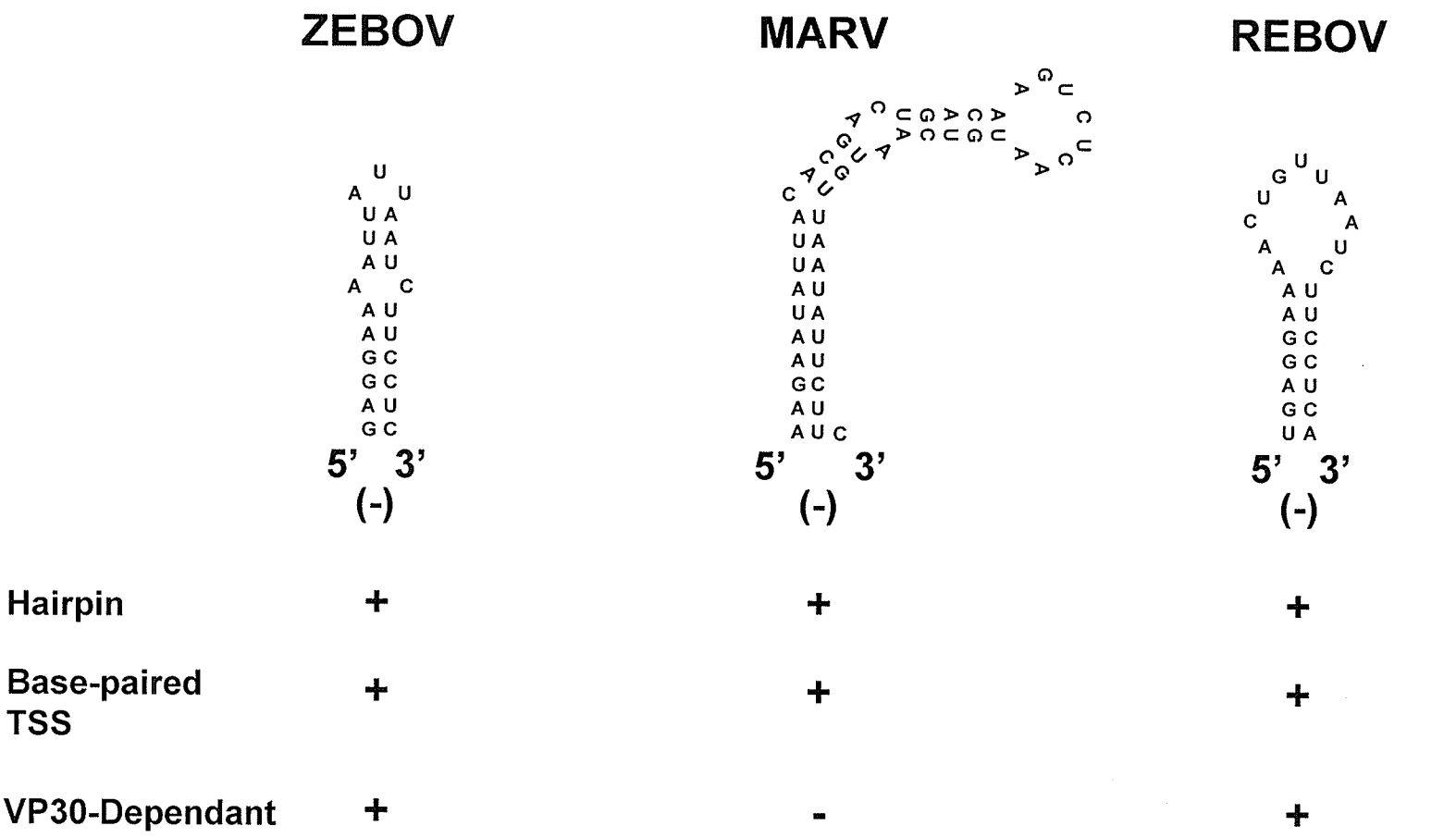



**Figure 31. RNA structure predictions for hairpin formation at the NP transcriptional start site.** The M-fold software package for RNA structure predictions was used to identify the most probable folding in REBOV, ZEBOV and MARV minigenomes. Based on these predictions hairpin structures near the transcriptional start site (TSS) of the NP gene (shown in green) were identified and correlated to the transcription phenotype, with respect to VP30 regulation, of the relevant virus.

### 3.5.3 Requirements for VP30 in Heterologously Transcribed Minigenome Systems

Since the formation of hairpin structures in EBOV and MARV genomes is predicted to be remarkably similar, given the general lack of conservation among filovirus non-coding regions as discussed earlier, it was of interest to determine whether there might be protein-specific contributions from the MARV RNP complex that lead to VP30 independent transcription. For this purpose, the transcription of our available filovirus minigenomes for ZEBOV, MARV and REBOV by homologous and heterologous RNP complexes provided a convenient approach by which genome-specific contributions could be eliminated. We, therefore, transfected the REBOV minigenome along with the RNP components from each of REBOV, ZEBOV and MARV. Similarly, the MARV minigenome system was transcribed using RNP complexes from either REBOV or MARV, and our collaborator Thomas Hoenen (Philipps Universität Marburg) transfected the ZEBOV minigenome with either ZEBOV or MARV helper plasmids. Based on reporter activity from each of these experiments it was apparent that transcription by MARV RNP complexes was independent of VP30 regardless of the genomic template (Figure 32 A, B and C). Similarly, transcription by EBOV RNP complexes required the presence of VP30 regardless of the origin of the template (Figure 32 A, B and C). Thus, the independence of MARV transcription on VP30 cannot be solely explained due to regulation by genomic hairpin structures, but seems to rely on some contribution of the RNP complex to overcome the need for an anti-terminator function.

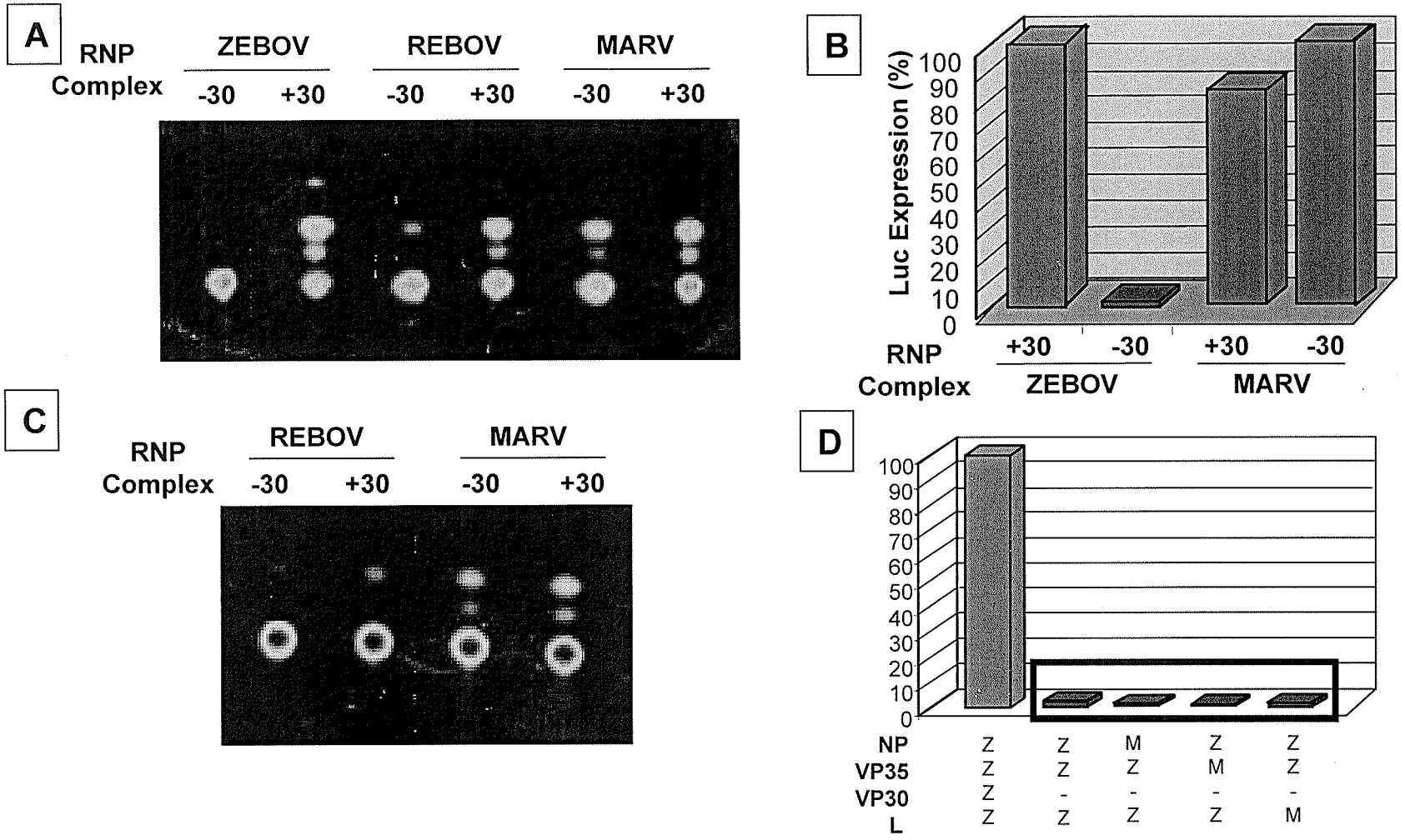



**Figure 32. Regulation of heterologous minigenome transcription by VP30. (A) REBOV minigenome.** The REBOV CAT minigenome was transfected along with all RNP components or in the absence of VP30 (+VP30,-VP30) using RNP plasmids for either REBOV (R), ZEBOV (Z) or MARV (M). After 48 h reporter activity was determined by CAT assay. **(B)** ZEBOV minigenome. The protocol in (A) was repeated using the ZEBOV Luc reporter plasmid and ZEBOV or MARV RNP complexes with or without VP30. Reporter activity was determined by luciferase assay. **(C) MARV minigenome.** The protocol in (A) was repeated using the MARV CAT reporter plasmid and REBOV or MARV RNP complexes. **(D) Complementation of VP30 function by MARV RNP components.** The ZEBOV Luc minigenome was transfected along with all ZEBOV RNP components or in the absence of VP30. In addition, individual MARV RNP components were substituted for their ZEBOV counterparts. Reporter activity was determined by luciferase assay.

### 3.5.4 Complementation of VP30 Function by MARV Proteins

In order to determine if any specific component of the MARV RNP was responsible for this difference in regulation, individual components of the EBOV RNP complex were replaced by their MARV counterparts. For these experiments the ZEBOV minigenome system was used, since this system relied on the detection of lucifersase as readout and as such was the most sensitive of the available systems, as well as providing a quantitative readout, which can potentially provide better discrimination of modest changes in reporter activity. Thus, ZEBOV minigenome components were transfected with or without VP30, and either NP, VP35 and L from MARV were substituted for their ZEBOV homologues in order to look for restorations of transcription. Unfortunately none of the individual MARV proteins were able to restore a significant level of function to the ZEBOV RNP complex. (Figure 32 D).

## 3.6 Inhibition of Viral Transcription/Replication Using shRNAs

One very exciting application of minigenome systems that has been recently described is their potential use for the screening of antiviral compounds that inhibit transcription and/or replication (Hass et al., 2004). The utility of the Lassa minigenome system could be used in this respect to clearly show the effect of both ribavirin and interferon-α in inhibiting viral transcription/replication (Hass et al., 2004). Therefore, we were interested to determine if the REBOV minigenome could be applied in a similar fashion, focusing on the identification of potent siRNA target sites for inhibition by RNA interference.

### 3.6.1 Inhibition of REBOV Minigenome Activity by shRNAs

Once a series of potential siRNA target sites within the non-coding 3' and 5' viral non-coding regions were identified (Table12, Figure 14) and corresponding shRNA expression vectors generated, these constructs were screened for function in our Pol I REBOV CAT minigenome system. The shRNAs were supplied at various concentrations and monitored for their ability to decrease reporter activity, indicative of inhibition of viral transcription/replication. While the shRNA targeting nucleotides 18295-18345 in the L untranslated region was able to substantially decrease reporter expression at all input masses between 2 and 0.25 μg, an shRNA targeting nucleotides 18714-18732 of the L untranslated region was only highly effective when 1μg or more was transfected (Figure 33A, compare lanes L UTR (18295) and L UTR (18714-18732)). In light of the apparent difference in potency of the different target sites, only the more potent of the two shRNAs was used in further testing.

### 3.6.2 Inhibition of REBOV Infection by shRNAs

In order to determine whether potential target sites validated through the use of our minigenome assay would prove successful in control virus infection in tissue culture, various amounts of shRNA plasmid, ranging from 0.5 to 6 μg, were transfected into VeroE6 cells for infection 24h later with REBOV. When RNA extracts from these cultures were analyzed for viral RNA load using strand specific RT-PCR for a region of the L open reading frame, it could be demonstrated that there was substantial reduction in transcript accumulation when higher input doses of shRNA plasmid (i.e. 3 or 6 μg) were used together with a lower MOI (MOI =0.005) of infecting virus (Figure 33B, upper

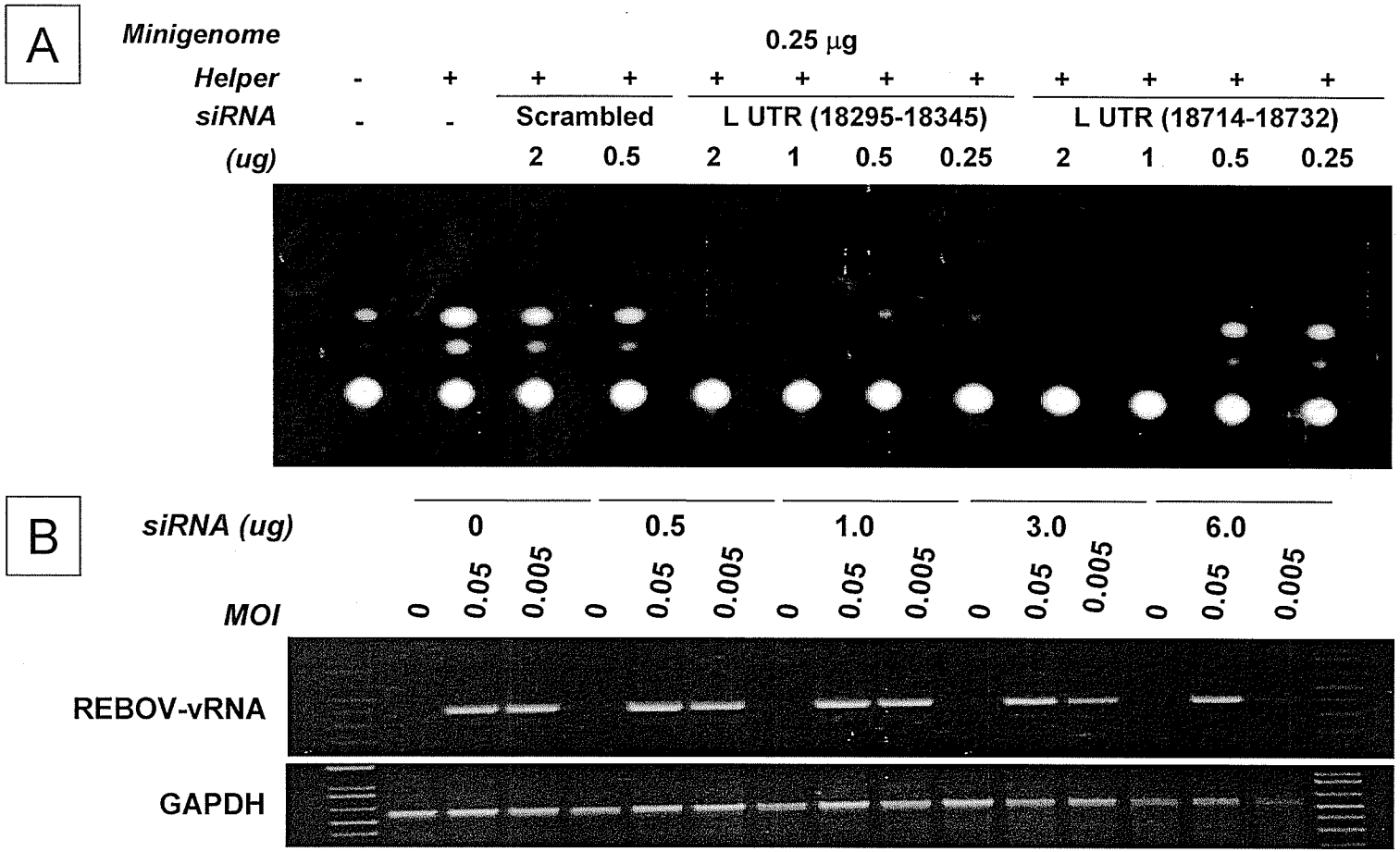



**Figure 33. Inhibition of reporter expression by REBOV-specific siRNAs. (A) Inhibition of REBOV minigenome activity by transient shRNA expression.** 293T cells were transfected with REBOV minigenome components along with various amounts of shRNA expression plasmids targeting the REBOV L untranslated region at nucleotides 18295-18345 or 18714-18732). Samples were incubated for 48h prior to harvest and assay for CAT activity. **(B) Suppression of vRNA accumulation in REBOV infected cells by transient shRNA expression.** VeroE6 cells were transfected with varying amounts of shRNA encoding plasmid directed against nucleotides 18295-18345 of the REBOV L 3' untranslated region. 24h later samples were infected with REBOV at an MOI of 0.05. Following extraction from infected cells vRNA was converted to a cDNA copy by RT using a strand-specific primer directed against the L open reading frame. Subsequently samples were amplified using REBOV L specific primers. Alternatively GAPDH mRNA was amplified from extracted RNA samples using gene specific primers.

panel). In order to standardize the RNA extracts for relative total RNA content, GAPDH was amplified as a representative cellular gene. No major deviations in RNA content were identified based on visual inspection of amplicon intensity following agarose gel separation and ethidium bromide staining (Figure 33B, lower panel).

### 3.6.3 Inhibition of ZEBOV Minigenome Activity by shRNAs

Given the successful identification of potent shRNAs capable of not only decreasing REBOV minigenome activity but controlling REBOV infection in tissue culture as well, we were interested to repeat these results using the ZEBOV minigenome system, since this virus species would not only be more clinically relevant with respect to antiviral development, but also provide the possibility to test shRNA function *in vivo* using a mouse model of ZEBOV infection (Bray et al., 1998). Therefore, potential siRNA target sites in the ZEBOV genome were similarly identified (Table12, Figure 14) and the corresponding sequences cloned into shRNA expression plasmids. When these plasmids were tested for their ability to decrease minigenome transcription, only one of the four tested target sequences, targeting nucleotides 193-213 of the NP untranslated region, was able to substantially reduce reporter activity at an input dose of 0.5 μg (Figure 34A). For this shRNA plasmid the effective concentration was further titrated over the region of 1.0 μg to 0.25 μg of input plasmid. This showed that only at doses of 0.5 μg or 1.0 μg could substantial decreases in reporter activity be observed (Figure 34B).

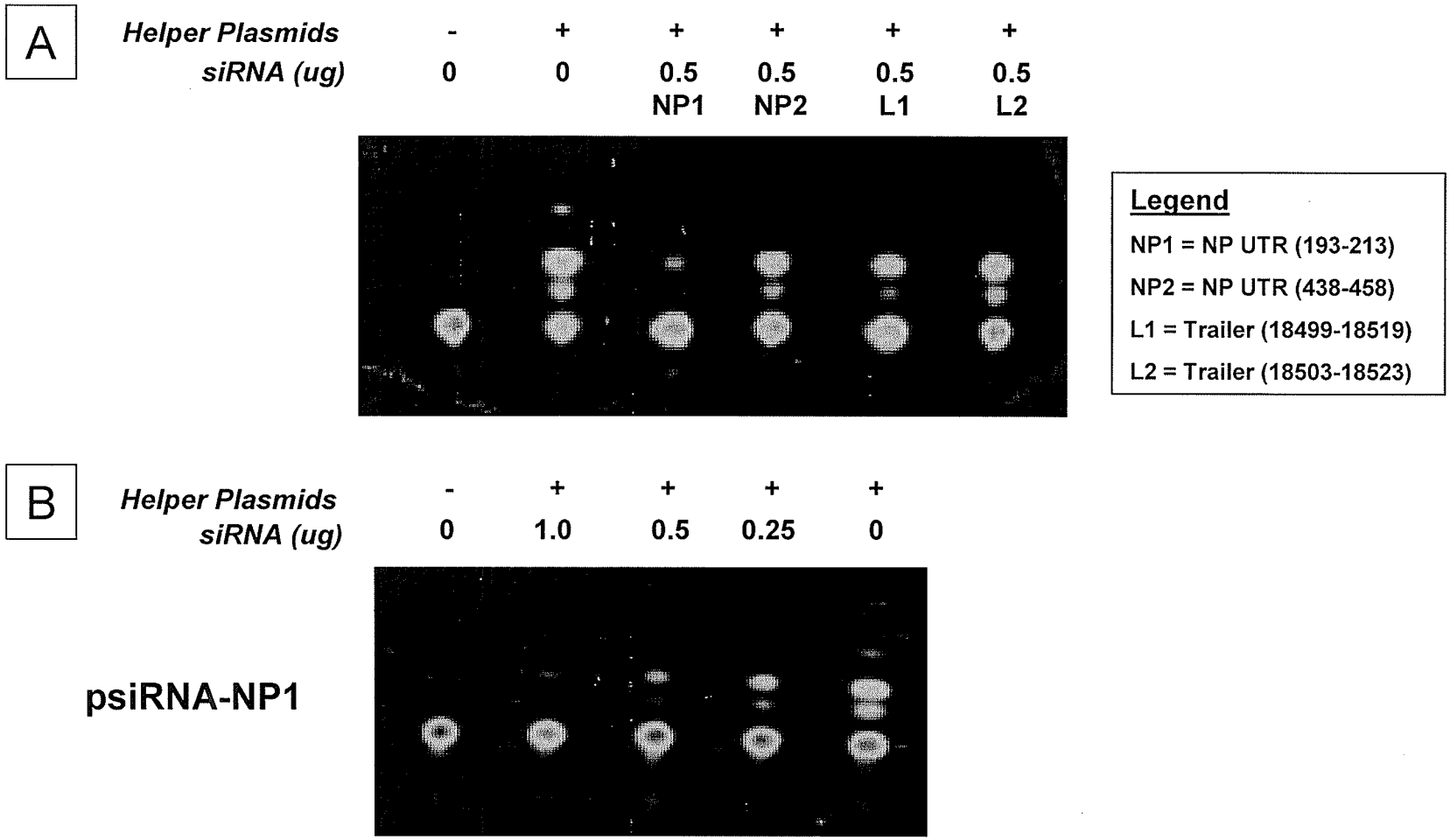



**Figure 34. Inhibition of reporter expression by ZEBOV-specific siRNAs. (A) Inhibition of ZEBOV minigenome activity by transient shRNA expression.** 293T cells were transfected with ZEBOV minigenome components along with various amounts of shRNA expression plasmids targeting the ZEBOV NP or L untranslated regions at the sites indicated. Samples were incubated for 48h prior to harvest and assay for CAT activity. (B) **Titration of ZEBOV minigenome inhibition by transient expression of the NP1 shRNA.** The protocol described in (A) was repeated using various amount of the NP1 shRNA expression plasmid.

### 3.6.4 Inhibition of ZEBOV-GFP Infection by Transient shRNA Expression

The most potent siRNAs against ZEBOV, targeting nucleotides 193-213 of the NP untranslated region, was selected for further analysis in a tissue culture model of ZEBOV infection. For this assay a GFP-expressing ZEBOV was used. This virus, created in our lab and kindly provided by Steven Theriault, expresses GFP from a separate open reading frame, inserted between NP and VP35, during virus transcription and was used in place of wild-type ZEBOV (Theriault, 2006). Due to the GFP expression, infection with this virus can be easily detected by either UV microscopy or FACS analysis, however, only a very slight attenuation in the kinetics of virus growth as a result of the additional open reading frame are observed (Theriault, 2006).

#### 3.6.4.1 Lipid Transfection of 293T cells

Since shRNA delivery is most easily accomplished using lipid transfection reagents, testing of shRNA function was initially attempted using 293T cells, which are highly transfectable and should, therefore, allow delivery of the shRNA plasmid to the majority of cells in the population. However, following infection with ZEBOV-GFP at an MOI of 0.05 it was observed that no detectable virus transcription was occurring, as determined by FACS analysis, regardless of whether the cells were transfected with the ZEBOV-specific shRNA plasmid, a scrambled shRNA plasmid or no shRNA plasmid at all (Figure 35A, left panel). Thus it appears that 293T cells are not sufficiently susceptible to infection for ZEBOV for use in these assays.

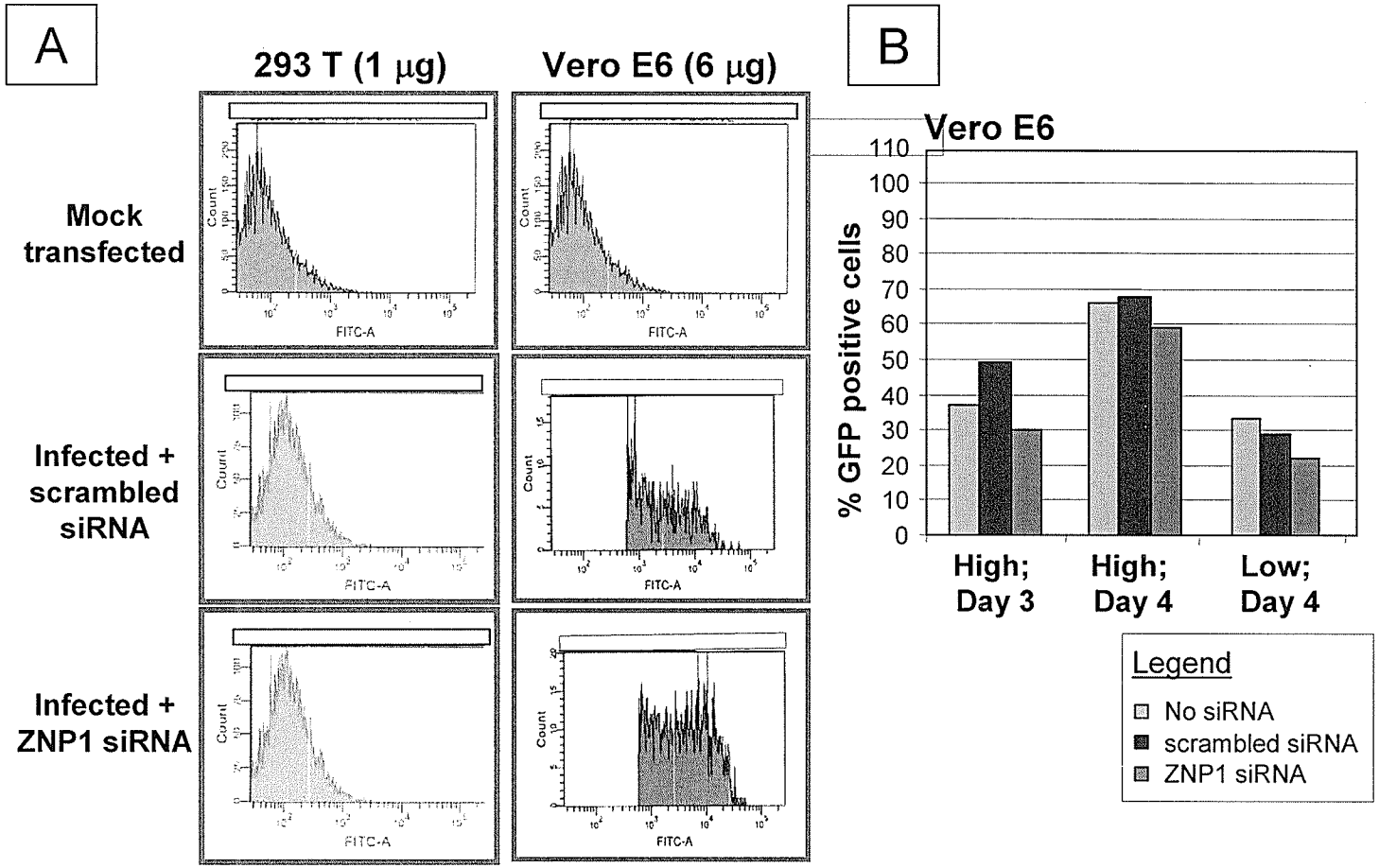



**Figure 35. Transient delivery of ZNP1 shRNA (A) Lipid mediated transfection.** ZEBOV specific shRNA expression plasmid or a scrambled control were transfected into 293T cells in 1 μg amounts or into VeroE6 cells in 1, 3 or 6 μg amounts, using TransIT-LT1. After 24 h cells were infected with ZEBOV-GFP at a MOI = 0.05 and after a further 4 days cells were analyzed by FACS for GFP expression. **(B) Electroporation.** 10 μg of ZEBOV specific shRNA expression plasmid or a scrambled control were transfected into VeroE6 cells by electroporation. After 24 h cells were infected with either MOI = 0.05 or 0.005 of ZEBOV-GFP and incubated for a further 3 or 4 days, as indicated. Cells were then analyzed by FACS for GFP expression.

### 3.6.4.2 Lipid Transfection of VeroE6 Cells

Since transfection of 293 T cells did not prove efficient an alternative approach was to attempt to demonstrate decreased transcription using a cell line known to be highly susceptible to ZEBOV infection. For this purpose VeroE6 cells were chosen. Following transfection with 1, 3 or 6 μg of shRNA expression plasmid or a scrambled shRNA vector the cells were infected at an MOI = 0.005 and analyzed for virus transcription. While GFP expression, indicating successful virus infection was observed, there was no apparent effect of shRNA treatment even at 6μg of input shRNA plasmid (Figure 35A, right panel). In fact, it appears that levels of GFP expression were slightly higher when cells were transfected with large amounts (i.e. 6 μg) of plasmid (Figure 35A, right panel). These data suggest that, consistent with the low transfection efficiency of VeroE6 cells, delivery of shRNA plasmid into target cells was not efficient using this procedure.

### 3.6.4.3 Electroporation of VeroE6 Cells

In order to overcome this barrier for plasmid delivery into VeroE6 an electroporation protocol was employed. Using this approach 10 μg of plasmid DNA, encoding either a ZEBOV-specific shRNA targeting the 3' NP untranslated region at nucleotides 193-213 (ZNP1)(Table 12) or a scrambled shRNA, was transfected per 6 well plate. These wells were infected 24 h later with an MOI of either 0.05 (high MOI) or 0.005 (low MOI) and analysed by FACS analysis for changes in GFP expression. It was observed that ZEBOV-specific shRNA plasmid transfection was able to mediate a decrease of 5-15 % at either MOI over a range of time points from 2 to 4 d post-infection

(Figure 35B). Based on this data the limiting step for shRNA function in VeroE6 cells seems to be successful delivery of the plasmid.

### 3.6.5 Inhibition of ZEBOV-GFP Infection by Stable shRNA Expression

#### 3.6.5.1 Generation of Stable shRNA Expressing Cell Lines

In order to overcome the limitations of available delivery methods for plasmid transfection in VeroE6 cells, stable cells lines were constructed. Selection of cells containing the shRNA expression plasmid following electroporation was facilitated by the presence of a neomycin resistance marker in the psiRNA vector backbone. In order to identify a concetration of Geneticin suitable for selection of resistant VeroE6 cells, non-transfected, and thus susceptible cells, were maintained in DMEM containing various concentrations of Geneticin over a period of 10 days and the viability of the cells was determined. Based on this experiment the concentration of Geneticin that was found to result in 90% mortality in susceptible cells in 10 days was 400 μg/mL, while the concentration for 50% susceptible cell death was 200 μg/mL (Figure 36A). These concentrations were used for selection and maintenance of resistant cell clones, respectively. Following isolation of 18 resistant cell clones it was necessary to identify those clones that were capable of expressing the shRNA at a level sufficient to mediate decreases in reporter activity for further testing. To do this the components of the T7-luc ZEBOV minigenome system were transfected into the cell clones and the resulting reporter activities determined, in collaboration with Thomas Hoenen (Philipps Universität Marburg).

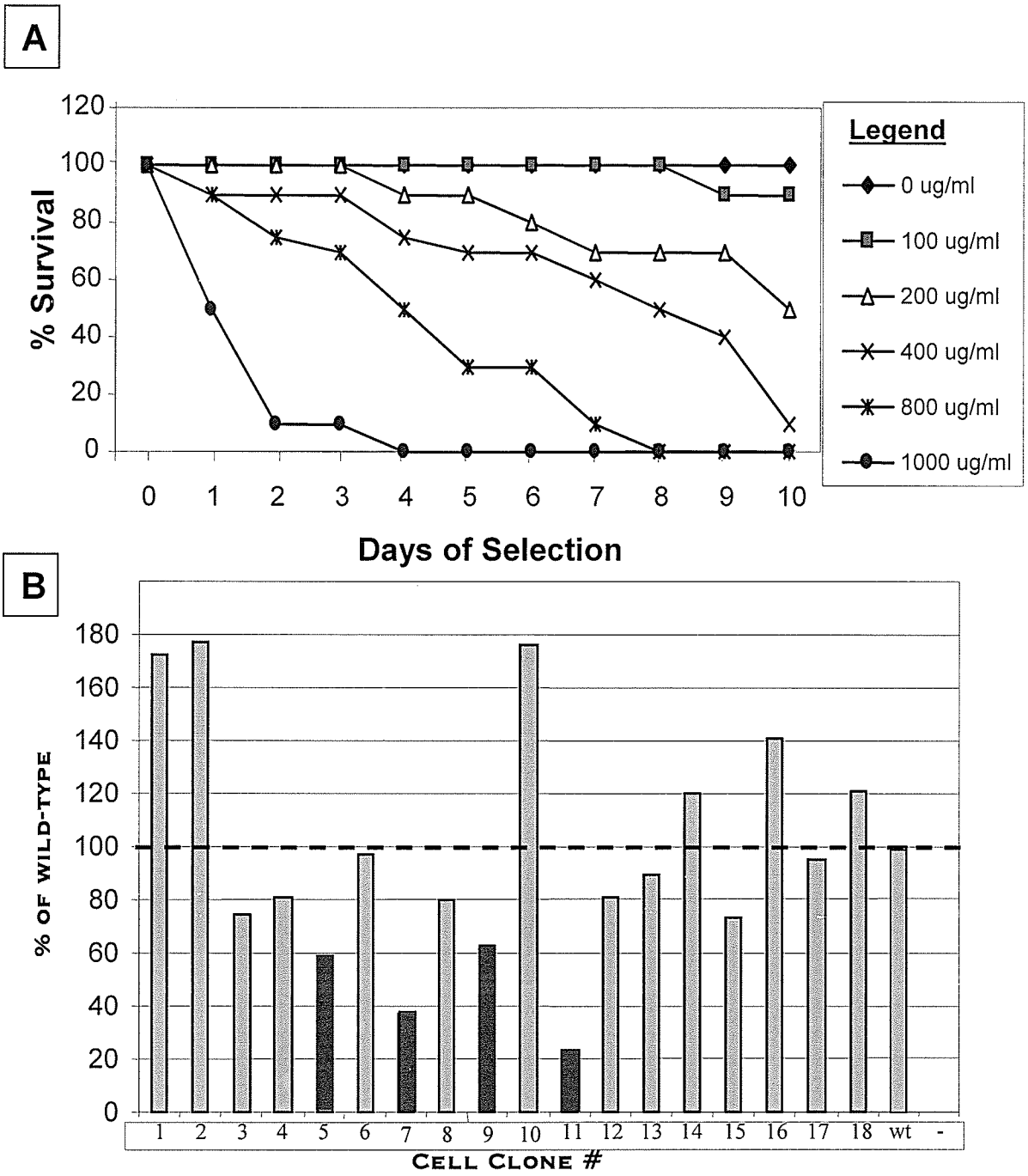



**Figure 36. (A) Survival of VeroE6 cells under Geneticin selection.** VeroE6 cells were cultured for 10 days, with media changes every 3 days, in various concentrations of Geneticin. Survival was assayed by visual inspection. **(B) Effects on minigenome transcription/replication as a result of shRNA expression in stable cell lines.** The ZEBOV Luc minigenome was transfected along with all RNP components into stable shRNA expressing cell lines. After 48 h reporter activity was measured by luciferase activity and standardized to the activity in wild-type VeroE6 cells which had been similarly transfected.

The cells clones that resulted in the largest decrease in reporter activity were #5, #7, #9 and #11 (Figure 36B). However, data from FACS analysis indicated that cell clones #7 and #9 displayed enhanced autofluorescence in their uninfected populations (data not shown), inconsistent with what is seen in wild-type VeroE6 cells, and, therefore, were excluded from further study.

### 3.6.5.2 Inhibition of GFP Expression by Stably Expressing shRNAs

With the remaining two cell clones, #5 and #11, that were able to mediate significant decreases in reporter activity, it was of interest to determine if, in the absence of barriers to siRNA delivery, any inhibition of virus infection would be observed. For this purpose the cell clones were each infected with either a low (MOI = 0.005) or a high (MOI = 0.05) MOI of ZEBOV-GFP and monitored for GFP expression. For high MOI infections, samples were taken at 3 and 4 days post-infection, while for low MOI infections samples were taken only at 4 days post-infection. When using a high MOI, clear decreases (between 15 and 45%), were seen on day 3, while at low MOI similar decreases (between 10 and 30%) were seen on day 4. Upon observation of the corresponding FACS histograms (shown for high MOI infection, 3 days after infection), a shift in GFP expression can clearly be seen (Figure 37B). This corresponds not only to the reduction in the percentage of GFP positive cells (indicated in Figure 37A) but also shows that among those cells that remain GFP positive there is a reduction in the level of GFP expression (Figure 37B). No significant decreases in the number of GFP expressing cells were seen by day 4 at high MOI (Figure 37A), however, closer examination of the histograms of GFP expression for this time point indicate significant changes in the

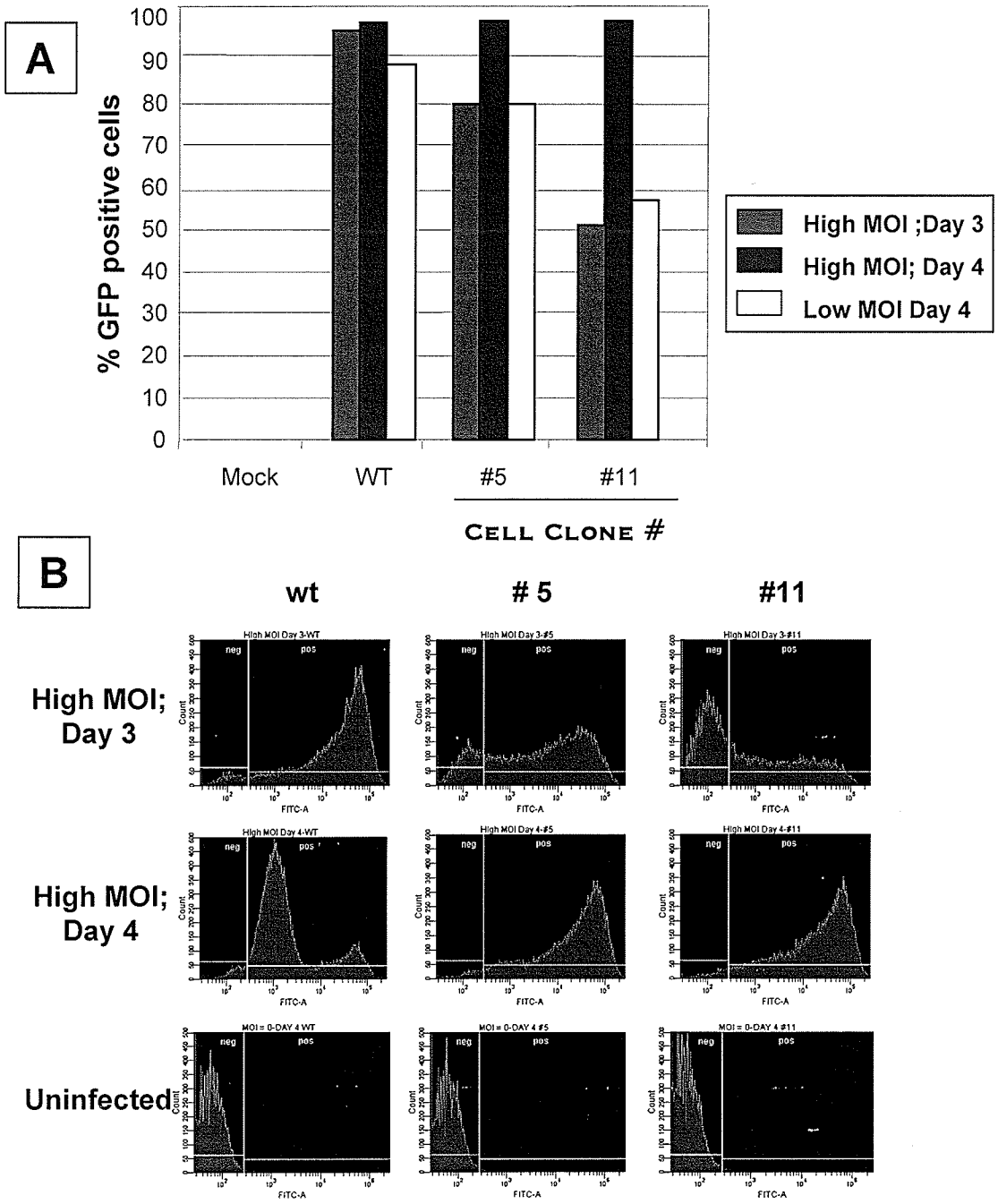



**Figure 37. Effect of stable shRNA expression on viral transcription (A) Percentage of GFP-expressing cells.** shRNA expressing cell lines were infected with GFP-ZEBOV at either a high or a low MOI and incubated for 3 or 4 days, as indicated. Subsequently the number of GFP-expressing cells was determined by FACS analysis and expressed as a percentage of the cell population. **(B) FACS analysis for GFP expression in shRNA stable cell lines.** The distribution of GFP positive cells analyzed above were plotted with respect to fluorescence intensity.

VeroE6 cell populations due to infection, not observed in the shRNA expressing cell lines (Figure 37B). This sudden decrease in GFP expression in previously highly expressing cells suggests deteriorating health and associated protein host shutdown in these cells due to infection.

### 3.6.5.3 Cytopathic Effects in ZEBOV Infected Stably shRNA Expressing Cell Lines

As an indication of decreased virus growth in infected cells, the cytopathic effects following ZEBOV infection in wild type VeroE6 cells, as well as the shRNA expressing cell clones, were analyzed. It was clearly observed that the decrease in GFP expression (Figure 38A, top panel) correlates with a decrease in cytopathic effect observed by bright field microscopy three days after infection with a high MOI of virus (Figure 38A, middle panel). This suggests that it is not GFP expression alone that is being modulated but that inhibition of virus growth is likely also occurring. No differences in cell morphology could be observed in the absence of virus infection (Figure 38A, lower panel).

### 3.6.5.4 Inhibition of NP Expression by Stably Expressed shRNAs

In order to assess the mode of action responsible for inhibition of GFP expression in shRNA expressing cell lines, the levels of NP expressed in cell clones #5 and #11 were determined following infection. If the mode of action is considered to be through the typically RNA interference pathways, then inhibition should involve targeting of the NP mRNA for degradation and, therefore, a corresponding decrease in protein levels. This can be clearly seen by western blot of infected cell lysates where cell clone #11 shows

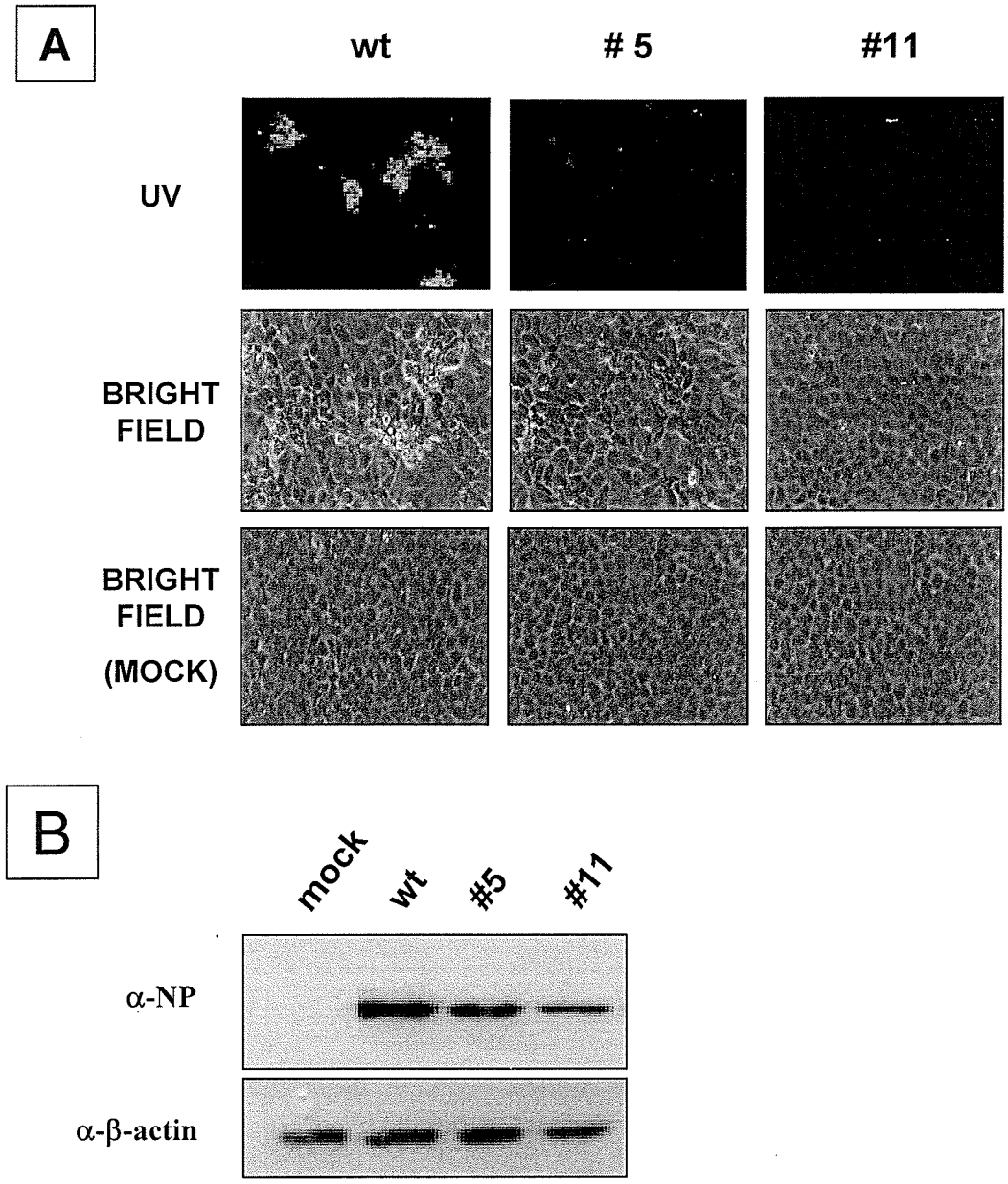



**Figure 38. Effect of stable shRNA expression on NP levels and the development of cytopathic effects in infected cells (A) Cytopathic effects in stable shRNA expressing cell lines.** VeroE6 cells of stable shRNA expressing derivatives were infected with MOI = 0.05 of ZEBOV-GFP or mock infected and, after 3 days, analyzed for cytopathic effects and virus infection by light and UV microscopy, respectively. **(B) Effects of shRNA expression on NP levels.** VeroE6 cells and stable shRNA expressing cell lines were infected with MOI = 0.05 of ZEBOV-GFP and incubated for 3 days. Cell lysates were prepared and examined by western blot for ZEBOV NP expression, using a monoclonal anti-ZEBOV NP antibody, or for actin expression using a monoclonal anti-actin antibody.

significantly reduced levels of NP expression and cell clone #5 shows more modest decreases compared to wild-type (Figure 38B). As a control, a western blot against actin was performed. This indicated consistent levels of actin present in all the cell samples (Figure 38B).

### 3.6.5.5 Inhibition of Progeny Virus Production by Stably Expressed shRNAs

Finally, in order to directly confirm an inhibitory action of shRNA expression on ZEBOV-GFP growth and resulting progeny virus titres, cells were infected with ZEBOV-GFP, as well as REBOV as a control for non-specific effects at any stage in the filovirus lifecycle.

#### 3.6.5.5.1 ZEBOV Infection

To assess for inhibition of ZEBOV infection, the shRNA expressing cell clones, as well as wild type VeroE6 cells were infected with MOI = 0.05 or MOI = 0.005 of virus and allowed to incubate for 3 or 4 days. At these time points supernatants were harvested and frozen for subsequent analysis. Once all supernatants were collected immunoplaque assays were performed on serial dilutions of virus supernatant in order to quantify virus output from infected cells. Reductions in virus progeny production of 1 - 1.5 logs, depending on the time point and infecting MOI (Figure 39A), were observed in shRNA-expressing cell clones.

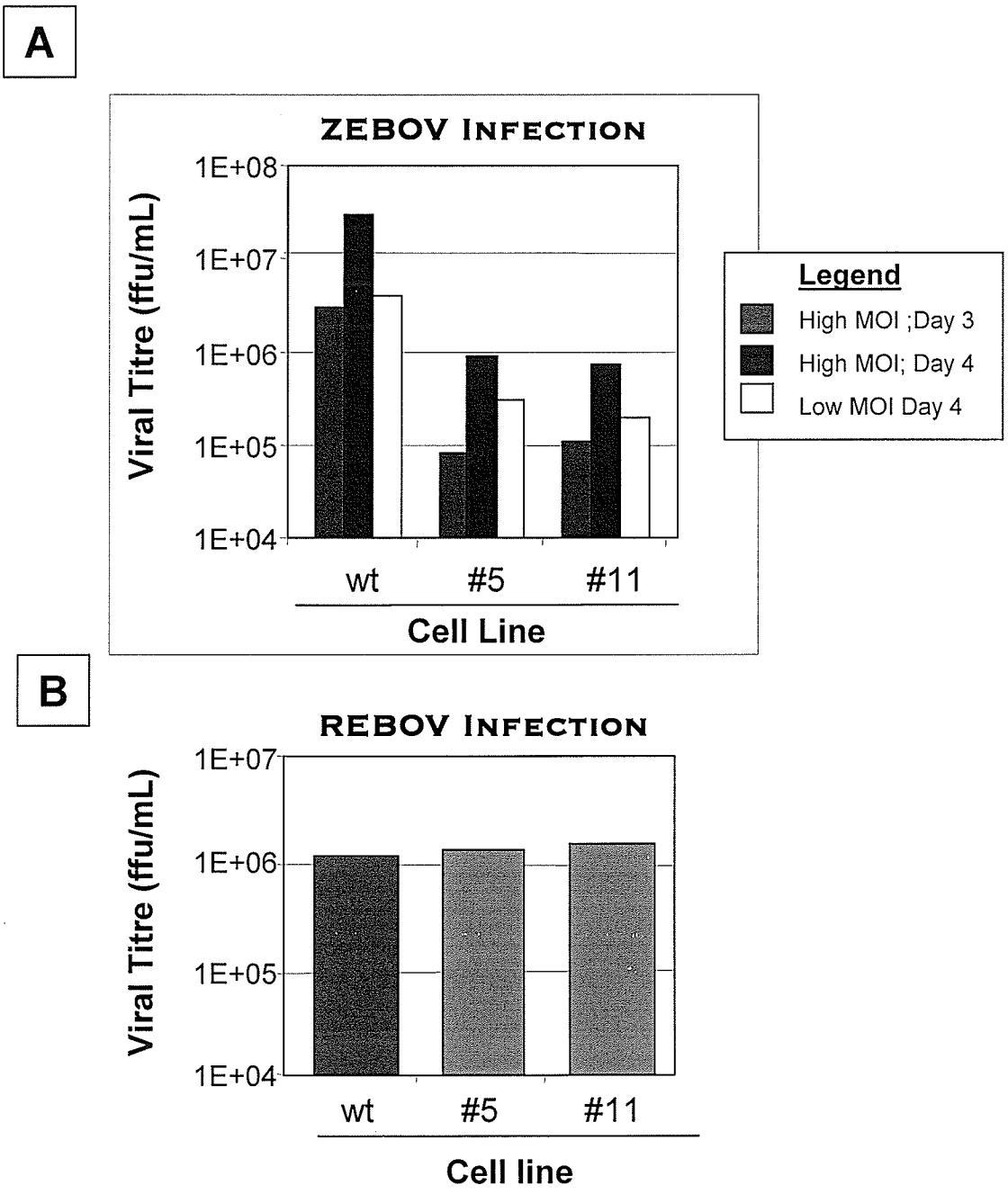



**Figure 39. Effect of ZNP1 siRNA expression on virus growth (A) Virus titre from ZEBOV-GFP infected cells.** VeroE6 cells or stable shRNA expressing cell lines were infected with a high or low MOI of ZEBOV-GFP and incubated for 3 or 4 days, as indicated. Supernatants were subsequently collected and used to infect fresh VeroE6 cells. Following infection these cells were overlayed with carboxymethyl cellulose and allowed to incubated for 5 days after which virus titre was calculated from the number of foci observed by UV microscopy. **(B) Virus titre from REBOV infected cells.** The protocol described in (A) was repeated using diluted REBOV stocks for infection of VeroE6 or shRNA expressing cell lines.

#### 3.6.5.5.2 REBOV Infection

To confirm that the cell clones being studied did not have any significant defects that would lead to impaired filovirus growth, irrespective of siRNA expression, the above procedure was repeated using REBOV infection. For detection of REBOV infection, dilutions of virus supernatants were analyzed by immunoplaque assay, using the anti-REBOV VP30 antibody described above. As can be clearly see in Figure 39B, there were no effects of stable ZEBOV shRNA expression on the growth of closely related filoviruses. Not only does this suggest that there are no underlying defects in the shRNA expressing cell clones, but it also indicates that non-siRNA related effect, such as immunological activation by siRNA expression, are not responsible for the decreases in virus titre.

### 3.6.6 Effects of shRNA Plasmid Treatment on Illness in ZEBOV Infected Mice

Since significant decreases in progeny virus titres were observed in infected cell cultures, it was of interest whether shRNA plasmid treatment would prove sufficiently potent to provide clinical benefit as a treatment *in vivo*. To assess this a preliminary experiment was undertaken in which, 5-6 week old female Balb/c mice infected with mouse-adapted ZEBOV were treated with three doses of 50μg of shRNA plasmid delivered at 24 h intervals. Treatment was begun either 24 pre-infection, 1 h post-infection or 24 h post-infection, and plasmid was delivered either in a 5% glucose solution, or complexed with linear PEI delivery agents conjugated to galactose, for enhanced liver uptake, or mannose, for enhanced macrophage and dendritic cell uptake.

In the mouse model weight loss is a very sensitive indicator of illness, thus weights from all infected animals were recorded for 11 days and plotted. The number of animals that lost more than 5 or 10% of their body weight during the course of infection was then graphed for analysis. When the number of mice that lost more than 5 or 10% body weight were observed, it was apparent that ZEBOV shRNA plasmid treatment alone is insufficient to protect animals in the challenge group from weight loss (Figure 40 A and B). However, it is also apparent that reduced number of animals experience weight loss if the plasmid is delivered prior to or shortly after infection (Figure 40A and B cf. lanes1, 6 and 11). In contrast, the delivery of a scrambled shRNA plasmid together with PEI could partially prevent weight loss, if the complexes were mannose conjugated for macrophage and dendritic cell targeting, or completely prevent weight loss, if conjugated with galactose for liver targeting. (Figure 40A and B). This effect by the scrambled shRNA plasmid was not seen to be dependent on the time that treatment was initiated (Figure 40A and B). Finally, when ZEBOV specific shRNA plasmids were delivered together with PEI they could completely prevent weight loss regardless of the target cell type or time of initiation of treatment (Figure 40A and B).

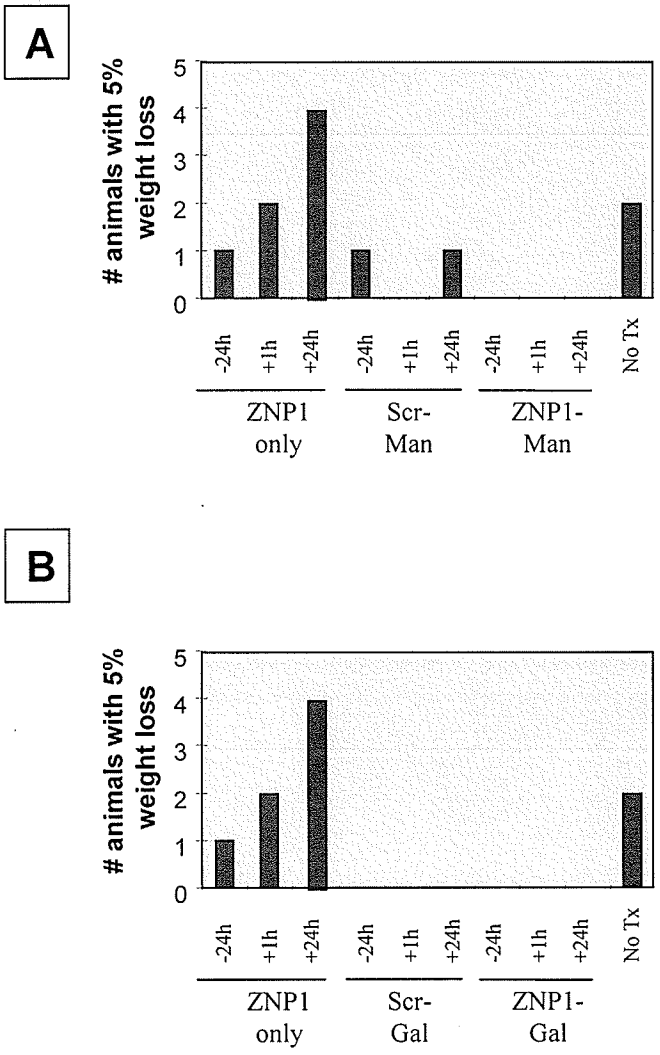



**Figure 40. Weight loss in ZEBOV infected mice. (A) PEI-Mannose delivery.** Groups of 6 mice were treated daily for 3 days starting either 24 h pre-infection, 1h post-infection or 24 post-infection with ZEBOV specific shRNA plasmid either alone or complexes with PEI conjugated to mannose. Mice were further infected on day 0 with 10 $LD_{50}$ of a mouse adapted ZEBOV variant and their weights monitored for 15 days. Based on these data the number of mice that lost >5% of their body weight were graphed. **(B) PEI-Mannose delivery.** Mice were infected and treated as above using PEI conjugated to galactose for shRNA delivery

# 4.0 Discussion

## 4.1 Project Background

Prior to this study, there was very little known about *Reston ebolaviruses* beyond the information contained in epidemiological accounts of the epizootics in the United States (CDC, 1990a, CDC, 1990b, Geisbert et al., 1992, Miranda et al., 1999, Rollin et al., 1999), and a few molecular reports characterizing the glycoprotein with respect to its glycosylation patterns (Feldmann et al., 1994), furin cleavage site (Volchkov et al., 1998) and the susceptibility of cell lines to infection with REBOV (Takada et al., 1997). However, interesting reports soon emerged indicating that, consistent with the lack of human disease associated with infection noted during the REBOV epizootics, REBOV infection in African green monkeys (Fisher-Hoch et al., 1992) and cynomolgus macaques (Jahrling et al., 1996) seems to be less virulent that infection with ZEBOV. Apart form the observation that the furin cleavage site in the REBOV glycoprotein, required for the generation of mature $GP_{1,2}$, deviates form the consensus sequence and might, therefore, be recognized and cleaved less efficiently (Volchkov et al., 1998), no basis for this difference was apparent. Further, a furin cleavage deficient recombinant ZEBOV showed no reduction in infectivity or loss of pathogenicity (Neumann et al., 2002a) (T. Geisbert, personal communication), which speaks against sub-optimal furin cleavage as a basis for altered pathogenic features in REBOV.

Therefore, we were interested in developing systems aimed at determining what molecular changes might contribute to the reported decrease in virulence of this unique filovirus species. Among the most powerful tools to carry out these kind of analyses are reverse genetics system, and these have increasingly been developed for other filoviruses

over recent years (Enterlein et al., 2006, Hoenen et al., 2006, Muhlberger et al., 1998, Muhlberger et al., 1999, Neumann et al., 2002a, Volchkov et al., 2001, Watanabe et al., 2004). Among these tools are minigenome systems, which allow analysis of viral transcription and replication in a BSL-2 setting, infectious VLP systems, which expand the minigenome system to include budding and subsequent entry of target cells and, finally, infectious clone systems, which allow virus rescue directly from cDNA and thus facilitate the study of genomic mutations in the context of the complete virus life cycle. However, confounding the development of such molecular tools was the absence of available sequence data, other than that for the glycoprotein (GP) gene [Accession number U23152], prior to the start of this study. Therefore, in order to undertake any molecular investigation, and in particular the development of reverse genetics, it was first necessary to obtain information about the viral genome. In addition to providing pre-requisite molecular information, it was also expected that this data would provide insight into elements of potential significance for pathogenesis, which could be analyzed in future studies via reverse genetics. With the availability of the necessary molecular data, the development of minigenome systems for REBOV was then undertaken with the expectation that the analysis of these systems would provide fundamental insight into the transcription and replication of REBOV, and possibly a link between these functions and pathogenesis. Further, it was believed that these systems would be well suited to answer outstanding questions in the field regarding these processes. In particular, while it was observed by Muhlberger *et al.* (Muhlberger et al., 1999) that VP30 functions as an Ebola specific transcription factor, by preventing early termination of NP mRNA synthesis (Weik et al., 2002), the absence of any such requirement for VP30 in MARV

transcription has never been explained. This is despite a number of lines of evidence, including conservation of genomic elements related to transcription/replication and identical protein functions between the genera, which suggest that these processes in EBOV and MARV are fundamentally very similar. Finally, it was expected that the information obtained regarding REBOV transcription and replication through the development and optimization of a minigenome system for REBOV would be valuable in ensuring proper design of more complicated reverse genetics tools for this virus in future.

In addition, due to the extreme virulence of many filoviruses species and the increasing frequency of outbreaks of these viruses, as well as increasing concern about the potential of these viruses to be used as biological weapons (Borio et al., 2002, Bray, 2003, Groseth et al., 2005, Leffel & Reed, 2004), the need for effective prophylactic and therapeutic measures is becoming increasingly urgent. One potential application of reverse genetics to this field is drug screening. In particular, minigenome systems have the potential to be used as high-throughput screening systems for potential antiviral compounds targeting transcription and/or replication in a BSL-2 environment. This potential was partly realized during the recent development of a minigenome system for Lassa virus, where IFN-α and ribvarin were shown to inhibit transcription, and thus reporter output (Hass et al., 2004). However, these systems have yet to be applied to the identification of novel inhibitors of transcription/replication. Therefore, it was of further interest to determine whether filovirus minigenome systems could be applied in this fashion, with the expectation that their use would lead to the identification of potent inhibitors of transcription/replication that would have activity not only in the minigenome system but against virus infection as well. One antiviral approach that has shown

promise in a number of viruses, including Hepatitis B virus, Foot and Mouth Disease Virus, Japanese Encephalitis Virus, West Nile Virus and Influenza virus (Bai et al., 2005, Murakami et al., 2005, Palliser et al., 2006, Tompkins et al., 2004, Uprichard et al., 2005), but which had not yet been examined for efficacy against filoviruses, was the use of siRNAs. Therefore, the final goal of this study was to use filoviruses minigenome systems to identify functional siRNAs and test them in cell culture and, if warranted, existing animal models of infection, for their ability to inhibit virus infection and thus therapeutic potential.

## 4.2 Genome Sequencing

Sequencing of the REBOV genome (strain Pennsylvania) was begun using previously developed diagnostic primer sets (Sanchez & Feldmann, 1996, Sanchez et al., 1996) [Appendix A] for amplification of small (~450 bp) targets from the REBOV NP, GP and L genes. Based on the sequencing of these products sufficient data could be obtained to facilitate a "primer walking" approach to sequencing the remaining genome, excluding the genome ends, which were amplified by genome end ligation and PCR amplification over this junction. Based on the full-length genome sequence it was apparent that this strain showed a high degree of homology to the concomitantly analyzed Philippine strain of REBOV (Ikegami et al., 2001)(Figure 17; Table 15). This was perhaps unsurprising, as all REBOV strains, with the exception of the Philippine strain, which was isolated directly from the source facility, were imported into the United States or Italy from a single primate export facility in the Philippines (CDC, 1990a, CDC, 1990b, Miranda et al., 1999). The limited genetic differences, 1-2% at the nucleotide

level, between these REBOV strains (Table 15) would, therefore, be in accordance with data reported for ZEBOV strains (Sanchez et al., 1996, Volchkov et al., 1997) and the finding that transmission within outbreaks results in few if any genetic changes (Leroy et al., 2002, Rodriguez et al., 1999).

In comparison to ZEBOV (strain Mayinga), conservation could be found within the open reading frames, the 3' leader and 5' trailer region and the transcriptional signals, whereas the non-coding and intergenic regions did not show any homology (Figure 17). While surprising, this clearly supports the classification of REBOV as a distinct species within the genus *Ebolavirus* and supports the hypothesis of a separate evolution of REBOV, consistent with its distinct geographical localization. Further comparison to the MARV genome indicates only short regions of conservation within some of the protein open-reading frames, in addition to conservation of the genome termini (Figure 17). These findings clearly reflect the relationship of the different viruses within the family *Filoviridae*, but also imply an important role for these conserved regions. In particular, the strong conservation of the 3' leader and 5' trailer regions at the ends of the genome indicates a vital role in the virus life cycle, a finding that is supported by their role as the minimal necessary signals for transcription and replication of monocistronic minigenomes (Muhlberger et al., 1998, Muhlberger et al., 1999). Based on this function these sequences should provide the signals for polymerase entry, encapsidation by NP, and packaging, thus their conservation between related virus species and even genera was not surprising.

In contrast, the situation with other elements believed to be important for transcriptional and replication was not as clear. In general, the sequence of

transcriptional start and stop elements was highly conserved, not only between EBOV species (Table 14) but in comparison to MARV as well. In comparison to ZEBOV, differences were found only in the last base of the transcriptional stop signal of the NP, VP30 and VP40 genes (Table 14). However, these variations occur at the position of a potential $6^{th}$ uridine residue. Since the remaining REBOV and ZEBOV genes demonstrate only 5 uridine residues, it appears that 3'-UAAUUCU$_{(5)}$-5' is a more accurate consensus sequence for the transcriptional stop signal of genes 1 to 6 of all Ebola viruses, rather than 3'-UAAUUCU$_{(6)}$-5' as has been proposed previously (Ikegami et al., 2001, Sanchez et al., 1993). However, it is still possible that the length of the uridine stretch (5 or 6 uridine residues) could influence the polyadenylation of the viral messenger RNA and, thus, play a subtle role in the differential regulation of filovirus transcription. Alternatively, it is possible the number of U residues in the upstream transcriptional stop sequence may impact on initiation at the downstream start site, as has been shown for VSV (Hinzman et al., 2002), although it is difficult to predict what effect the longer intergenic regions of filoviruses might have on this effect.

It is also interesting to note that REBOV displays the same non-consensus transcriptional stop signal following the L open reading frame that is seen in the ZEBOV genome. While evidence from the structure of VSV transcriptional stop elements would suggest that this change may not be important (Barr & Wertz, 2001) since it conserves the presence of an A/U-rich stretch upstream of the run of U residues, it is interesting that this alteration is maintained between different EBOV species, despite the lack of non-coding regions conservation otherwise seen.

In addition, comparison of the pattern of transcriptional start and stop sequences indicate that REBOV lacks a second transcriptional stop signal following the VP24 coding region found in ZEBOV (Sanchez et al., 1993). It remains to be shown what significance this might have for gene regulation, however, it has been suggested that this second stop signal might play a role in preventing the formation of bicistronic messenger RNA species containing both the VP24 and L open reading frames (Sanchez et al., 1993). Alternatively, the presence of a second transcriptional stop signal might play a role for reducing reinitiation at the downstream L transcriptional start through prolonged transcriptional pausing and/or replicase dissociation. If regulation of transcription and replication of filoviruses can be assumed to parallel that in VSV, the resulting decrease in polymerase expression may enhance viral growth (Meier et al., 1987).

Finally, comparison between REBOV and ZEBOV showed unexpected differences in the number of gene overlaps present in the genome. These gene overlaps are composed of the transcriptional stop signal of an upstream gene and the start signal of a downstream gene overlapping at a conserved UAAUU pentamer, which is an integral part of both signals, and are a common feature among filoviruses (Bukreyev et al., 1995, Feldmann & Kiley, 1999, Feldmann et al., 1992, Ikegami et al., 2001, Sanchez et al., 1993). Historically, their positions and numbers have been used as a criterion for differentiation among viruses of the two genera within the family *Filoviridae* (Feldmann & Klenk, 1996). However, it is apparent that, while similar in its pattern of overlaps, REBOV is distinguished from ZEBOV, by the absence of a gene overlap between the glycoprotein and the VP30 genes (Figure 17A). In light of the impaired growth of REBOV in cell culture, the discovery of this alternative overlap arrangement in REBOV

might be considered to support a previous hypothesis that gene overlaps are a regulatory element for gene transcription (Sanchez et al., 1993). While a direct effect of gene overlaps on transcript levels has yet to be shown, it is easy to imagine how the direct juxtaposition of transcriptional stop and start signals could alter transcriptional attenuation at intergenic region sequence. This would certainly be consistent with findings in VSV that indicate a direct role for intergenic region length in regulating attenuation (Stillman & Whitt, 1998).

Similar to the situation with the non-coding regions, minor but provocative, and potentially significant, differences were observed in the protein open reading frames of the REBOV and ZEBOV as well. While there were many minor changes (i.e. single amino acid changes), whose impact is difficult to assess without a much more detailed knowledge of the protein structure/function relationship than is currently available for filoviruses, a few more dramatic changes were noted. In particular, it was observed that the carboxyl-terminal half of NP, was highly variable between the species of EBOV. This region has been previously reported to be less conserved among filoviruses and has been discussed for a potential role in the assembly process (Feldmann & Kiley, 1999). However, if NP-VP40 interactions are a driving force for budding, as is widely believed (Licata et al., 2004), it is difficult to rationalize the variability of this region with the high degree of conservation in VP40. Indeed VP40 is the third most conserved protein (74.0% amino acid identity; compared to 74.2% for L and 80.1% for VP24) based on a REBOV/ZEBOV comparison. Likely, this conservation is due in part to the abundance of highly ordered secondary structures within VP40 (Dessen et al., 2000a, Dessen et al., 2000b) as well as wide variety of interactions that VP40 undergoes, including two forms

of homo-oligomeriztion (Gomis-Ruth et al., 2003, Ruigrok et al., 2000, Scianimanico et al., 2000), interaction with RNA (Gomis-Ruth et al., 2003), and interaction with several host protein (Nedd4, Vps4, Tsg101) (Harty et al., 2000, Licata et al., 2003, Martin-Serrano et al., 2001, Timmins et al., 2003, Yasuda et al., 2003) as well as, presumably, with other viral proteins. Despite this overall high level of conservation it is then surprising that the carboxyl-terminal end of REBOV VP40 contains an extension of 5 amino acids (QNSYQ) compared to ZEBOV. Based on our rather extensive knowledge of VP40, compared to other viral proteins, it can be suggested that, since the C-terminal domain of VP40 is critical for membrane interaction, presumably through large surface exposed hydrophobic patches (Dessen et al., 2000a, Dessen et al., 2000b), the addition of this short but relatively hydrophilic sequence may have implications for budding in REBOV. Additional changes included those at the amino-terminal end of VP35, where the first 11 amino acids were missing in a direct comparison, and the last 26 amino acids of VP30, which lacked substantial homology to ZEBOV and included a single amino acid truncation. However, to date no protein functions have been identified in these regions, and in the absence of a crystal structure, such as is available for VP40, speculation regarding the potential impact of these changes is difficult.

Overall, despite these subtle differences in the protein ORFs and elements important for transcriptional and replication, the high degree of conservation of both protein and genomic elements involved in transcription and replication suggests that these processes in REBOV are likely to be very similar to those of other filoviruses. This assumption, along with the availability of complete sequence data, and particularly

reliable data for the genome termini, provided the basis for our attempts to develop reverse genetics systems for REBOV.

## 4.3 Antibody Production

In addition to the absence of sequence data for REBOV, the availability of immunological reagents, including antibodies, for this virus were, and continue to be, extremely limited. Therefore, in order to address this need, and particularly to allow detection of RNP protein expression from eukaryotic expression vectors developed as part of the minigenome system, antisera were raised against bacterially expressed GST fusion proteins, containing the VP35 ORF, the VP30 ORF or peptide sequences from NP or L.

Based on detection in western blot and IFA, working antibody dilutions were established for both of these assays using the VP35, VP30 and NP peptide antisera. For the anti-NP2 antiserum these were determined to be 1:200 (IFA) and 1:2000 (WB), for anti-VP35 they were 1:400 (IFA) and (1:4000) and for anti-VP30, 1:1000 (IFA) and 1:4000 (WB). Presumably the lower dilution of antibody required when using the anti-NP2 antiserum is due to the limited number of potential targets generated after immunization with the short peptide sequence. In contrast, the VP35 and VP30 antibodies, which were raised against whole protein targets, presumably contain a larger pool of antibodies against spatially distinct sites, which would allow many antibodies to bind on any given molecule of protein, thus enhancing the signal. Unfortunately, neither of the anti-L peptide antisera were able to detect the protein when expressed in its entirety. While this was not completely unexpected given that a number of attempts to

generate anti-L antisera by others in the field have also failed (H. Feldmann, personal communication; H. Ebihara, personal communication), the absence of a functional antibody meant the additional challenge of finding an alternative method to detect L expression.

## 4.4 RNP Complex Protein Localizations and Function

In addition to validating our anti-REBOV antisera, patterns of localization could also be established for singularly expressed REBOV NP, VP35 and VP30 based on these IFAs. Consistent with localizations previously reported for the corresponding MARV proteins (Becker et al., 1998) staining indicated that NP is present in cytoplasmic inclusions, while both VP35 and VP30 were diffusely localized throughout the cytoplasm (Figure 20B). Although not previously documented for any EBOV species, it is not surprising that these proteins would display similar localizations to that of their homologues in MARV, since these proteins are presumed to serve the same functions in both viruses. One further observation made by Becker and colleagues regarding RNP complex protein localization in MARV is that interaction of VP35 or VP30 with NP during coexpression is sufficient to mediate relocalization of those proteins to cytoplasmic inclusions (Becker et al., 1998). This provided us with a further test of authenticity since, as mentioned previously the interaction of VP35 and VP30 with NP are critical for formation of the replicase complex (Becker et al., 1998). Indeed, coexpression of VP35/NP or VP30/NP showed localization of either VP35 or VP30 to inclusion bodies, consistent with interaction with NP (Figure 22). Perhaps more surprisingly, coexpression of VP35/L or VP30/L also showed localization of VP35 or

VP30 into inclusion bodies. This is in contrast to what was shown by Becker et al. using a flag-tagged L construct and suggests that REBOV L itself may be a driving force for inclusion body formation, although it remains unclear whether L alone would be localized to inclusions or whether interaction with VP35 or VP30 are necessary for this distribution. This difference from what has been previously reported for MARV may be due either to the presence of the flag tag affecting the distribution of L in these experiments, or the presence of coinfecting vaccinia virus, which has been previously shown to alter the expression of another viral protein, GP (Sanger et al., 2001). Alternatively, this may represent one of the few reported differences between the filovirus genera. It also cannot be ruled out that the formation of inclusion bodies by L is a by-product of its high level of expression from the pCAGGS plasmid and may, therefore, not accurately reflect what happens during viral infection where L is in relatively low abundance. However, in VSV, over-expression of L beyond physiologically relevant levels has been shown to inhibit virus replication (Meier et al., 1987), a phenomenon not observed in the REBOV minigenome system after transfection of L expression plasmid amounts equal to those used in these experiments (Figure 25), suggesting that such levels have not been exceeded and so normal protein function would be expected.

Despite these results, consistent with the authentic production of NP, VP35 and VP30, it remained necessary to confirm correct expression of L. In addition, a more definite and direct method of validating REBOV RNP protein function was desired. In the ZEBOV infectious clone system it was observed that virus rescue from this system, in contrast to what had been previously reported using the minigenome system (Muhlberger

et al., 1999) was possible using a MARV RNP complex (Theriault et al., 2004). Therefore, in order to evaluate authentic expression of the REBOV RNP proteins, each expression plasmid was substituted individually for its counterpart in the ZEBOV infectious clone system. In addition, pairwise substitutions were also performed to examine the possibility that certain protein-protein interactions might be highly specific and, therefore, not compatible with their ZEBOV counterparts. Finally, as a control, the entire RNP complex from REBOV was used to drive virus rescue. While rescue was shown to be possible with all studied protein combinations, clearly confirming their expression and function, more detailed analysis of the data also provided some insight into specificity of interaction within the RNP complex (Figure 23). Specifically, lower frequencies of rescue using combinations that included a heterologous source of NP or L indicated that there is a more stringent species specificity for these components. At least in the case of NP, this can be viewed as being consistent with the high species to species variation in the C-terminal half of this protein and its proposed role in as yet loosely defined species-specific functions (Feldmann & Kiley, 1999).

## 4.5 Minigenome Systems for REBOV

With the availability of the necessary resources to develop a REBOV minigenome system, consideration was given as to what approach should be employed to mediate transcription of the minigenome cassette. While all filovirus reverse genetics systems at that time were based on initial transcription by T7 polymerase (Boehmann et al., 2005, Enterlein et al., 2006, Muhlberger et al., 1998, Muhlberger et al., 1999, Neumann et al., 2002a, Volchkov et al., 2001, Watanabe et al., 2004), we elected to develop a

minigenome system for REBOV based on an alternative to this classical approach. Based on the success reported with several bunyavirus systems (Flick et al., 2003a, Flick et al., 2003b, Flick & Pettersson, 2001), minigenome cassettes were designed and constructed based on an initial transcription step mediated by RNA polymerase (Pol) I. It was anticipated that the use of a host polymerase, and thus elimination of the need to introduce an exogenous source of polymerase into mammalian cells, would overcome a significant barrier to efficient functioning of the minigenome system. Specifically, while introduction of T7 into mammalian cells can be achieved in a number of ways, the need to do so presents a potential limitation if the entire population of cells is not targeted, as is often the case with plasmid transfection or even stably expressing cells, which in our experience can exhibit much less than 100% expression. Minigenome transcription by Pol I would also bypass significant limitations of the T7-driven system relating to the production of authentic transcripts, since Pol I-mediated transcription produces non-modified transcripts, which have correct terminal sequences, lacking additional promoter or non-coded nucleotides. Historically, the tendency of T7 to incorporate additional 3' end residues has been dealt with by incorporation of a hepatitis delta virus (HDV) ribozyme sequence at the 3' end of the transcript. However, subsequent cleavage of transcripts by the HDV may be a major limiting step for efficient minigenome rescue as there are indications that cleavage occurs in only about 30% of ZEBOV minigenome transcripts (J. Modrof, personal communication). If assumptions regarding the role of correct secondary structural formation in the genome termini are correct, this implies that less than a third of T7 transcripts produced would go on to provide functional templates for mRNA production. While the Pol I system is now widely used for the artificial

generation of influenza A virus (Hoffmann et al., 2000a, Hoffmann et al., 2000b, Hoffmann & Webster, 2000, Neumann et al., 1999), one potentially limiting factor in the use of Pol I with cytoplasmically replicating viruses, such as the filoviruses, is the nucleolar localization of Pol I within host cells. However, since recent data indicate that this does not present a barrier to successful rescue of reporter activity from bunyavirus systems, which are also cytoplasmically replicating (Flick et al., 2003a, Flick et al., 2003b, Flick & Pettersson, 2001, Pinschewer et al., 2003), it was probable that this system could be equally successful when used to mediate filovirus minigenome transcription. Although, for the study of some viruses the host restriction demonstrated by Pol I could also prove problematic if cell lines from species whose promoter and terminator sequences have not been determined are required for study, this was also not a concern for filoviruses, which grow well in several human and non-primate-derived cell lines.

Indeed, following optimization of the REBOV Pol I minigenome system, it became clear that a Pol I-driven REBOV minigenome generates both a higher level of reporter activity and a higher signal-to-noise ratio than did a comparable T7-driven REBOV construct (Figure 27). Interestingly, attempts to increase the efficiency of T7-driven transcription, and thus the initial transcription of vRNA-like molecules, by the insertion of G residues at the +1, +2 and +3 positions relative to the T7 promoter and the minigenome cassette, had no effect on reporter activity (Figure 28). Indeed, slightly higher levels of reporter activity were observed with the construct lacking all 3 upstream G residues, indicating that the more significant effect of these additional nucleotides may be to disrupt interactions in the complementary terminal non-coding regions, thereby

impairing recognition by RNP complex proteins and subsequently transcription and replication. While this finding appears to violate what is known about the minimal sequence requirements for the T7 promoter, it is possible that the requirement for G residues in the +1-+3 positions as part of the minimal promoter sequence is already overcome in filovirus minigenome systems, since the viruses themselves contain G/C-rich termini (Figure 18) which might compensate for this function. These observations will be important in directing the design of an infectious clone system for REBOV, where optimization of individual steps may play a substantial role in contributing to an overall successful rescue system.

As with the bunyavirus systems, the mechanism by which export of Pol I transcribed minigenomes to the cytoplasm, where interaction with viral RNP complex proteins take place, might occur is unclear. However, the application of this system to two distinct families of cytoplasmically replicating viruses suggests either limited non-specific export of these transcripts or an as yet undescribed mechanism for export of these unmodified RNAs (Cullen, 2003). However, it must also be noted that transcription of minigenome RNAs by Pol I may in fact occur in the cytoplasm either during periods of mitotic nuclear membrane breakdown or immediately following Pol I translation but prior to nuclear localization. Regardless of this mechanistic ambiguity, it is clear that Pol I mediated transcription represents a simplified alternative for the development of minigenome systems that seems to be applicable to a broad range of viruses, including those in *Mononegavirales*. It is, however, important to note that the application of this system may, in many cases, be limited to the development of minigenome systems, since

it has yet to be demonstrated if the longer transcripts required for most infectious clone systems can be synthesized by Pol I and successfully exported from the nucleus.

In addition to providing a proof of concept for the development a REBOV minigenome system based on a comparatively novel transcription system, the availability of this system was instrumental in validating a number of assumptions that had been made regarding REBOV based on analogy with other filoviruses. This is of particular significance with REBOV, since it is expected that key differences between this and other more virulent filoviruses will exist, thus these assumptions cannot be taken for granted. The first minigenome system for a filovirus was established for MARV and using this system, it was determined that only three of the four RNP complex proteins (NP, VP35 and L), were needed for transcription and replication (Muhlberger et al., 1998). In contrast, however, transcription and replication of the ZEBOV minigenome required all four RNP complex proteins (NP, VP35, VP30 and L) (Muhlberger et al., 1998). Using the Pol I-driven minigenome system it was confirmed that, consistent with what was shown for ZEBOV, the rescue of reporter activity from REBOV minigenomes required the presence of VP30 (Figure 30). This finding is consistent with the idea that VP30 acts as an EBOV-specific transcriptional activator. In addition, the amounts of each RNP complex protein encoding plasmid that should be transfected for maximum reporter activity were very carefully worked out in the ZEBOV minigenome system (Muhlberger et al., 1999). Again, the findings with REBOV were very consistent; with the exception that larger amounts of L plasmid were seen to give slightly enhanced reporter activity. However, this finding was later confirmed in the ZEBOV minigenome system (Watanabe et al., 2004) as well. Finally, it was determined that, similar to other filoviruses, the 3'

and 5' non-coding regions contain the minimal necessary *cis*-acting signals for packaging of minigenome vRNAs (Figure 26). However, it remains to be seen if additional elements in the genome, particularly those near the termini in the NP of L ORFs, will contain signals that enhance the efficiency of packaging, such as has been shown for influenza virus (Fujii et al., 2003, Liang et al., 2005, Watanabe et al., 2003).

## 4.6 Comparative Transcription by Filovirus RNP Complexes

Filovirus transcription and replication have long been thought to be highly specific processes dependent on both protein-protein interactions as well as protein-RNA interactions (Muhlberger et al., 1998, Muhlberger et al., 1999). However, recent data generated, using the ZEBOV infectious clone system indicated that the RNP complex proteins of filoviruses can facilitate transcription and replication of templates derived from a virus of a different genus or species within the family (Theriault et al., 2004). It was originally considered that this might represent a difference in readout sensitivity between the two systems, although it was unclear whether the linear measurement of transcription events in the minigenome system or the replicating nature of the infectious clone system was a more biologically relevant test of protein function.

However, subsequent experiments using the Pol I REBOV and T7-MARV minigenome systems could confirm the findings using the ZEBOV infectious clone system, indicating that REBOV, ZEBOV and MARV RNP complex proteins can all mediate transcription in both these systems (Figure 29). While these data conflict with previous reports (Muhlberger et al., 1999), they clearly indicate that filovirus transcription and replication are neither strictly species- nor genus-specific, regardless of which system is used as a readout.

In addition to confirming the possibility of minigenome transcription/replication by heterologous RNP complex proteins, these experiments produced a surprising finding. It was observed that reporter gene expression was stronger with RNP complex proteins derived from ZEBOV and MARV than for REBOV, regardless of the phylogenetic relationship between the viruses from which the RNP complex and the RNA template were derived (Figure 29). Since the use of a single minigenome template together with multiple RNP complexes excludes the impact of genomic difference, these data then clearly suggest that viral virulence could be related to transcriptional efficiency of the polymerase complex. This is certainly consistent with the slower growth of REBOV in tissue culture and the delayed time to death in non-human primate models (Fisher-Hoch et al., 1992, Jahrling et al., 1996).

Further, while it might be expected that the viral RNA would have coevolved with its RNP proteins for efficient protein-RNA interaction, this finding is consistent with the rescue data for the ZEBOV infectious clone system using heterologous RNP complex proteins, which indicated that protein-protein interactions were more important for successful rescue than protein-RNA interaction (Theriault et al., 2004).

## 4.7 Regulation of Transcription by VP30

It is clear from past work with filoviruses (Muhlberger et al., 1998, Muhlberger et al., 1999, Watanabe et al., 2004, Weik et al., 2002) as well as other virus systems (reviewed in (Neumann et al., 2002b)), that minigenome systems provide powerful tools to study viral transcription and replication. Therefore, we were interested to apply our newly developed REBOV minigenome system, together with other existing systems, to

addressing an outstanding question regarding the regulation of transcription, that is: why should VP30 be required for the transcription of EBOV but not MARV? That this is in fact the case has been very clearly demonstrated by Muhlberger and colleagues (Muhlberger et al., 1998, Muhlberger et al., 1999) and was confirmed here (Figure 30). Further, it has been shown that in the context of EBOV transcription VP30 acts as an early anti-terminator for the transcription of NP, but not subsequently transcribed genes (Weik et al., 2002). Also, it was shown that the requirement for VP30 could be overcome by disruption of a hairpin structure upstream of the NP, and thus it was proposed that VP30 may be acting either to resolve this structure in the genome, a mechanism that is unlikely due to encapsidation of the genome by NP, or to prevent its formation in the nascent mRNA, which had been suggested to trigger premature termination (Weik et al., 2002). However, it remains unclear why a similar mechanism of regulation does not occur in MARV. Perhaps the simplest explanation for this phenomenon would have been the absence of the hairpin structure through which VP30 regulation acts. However, minigenome RNA folding predictions indicated that there may be a hairpin present in MARV which is in the same relative location in the genome, overlapping the NP transcriptional start site (Figure 31). Since the basis for this difference in transcriptional regulation was not immediately apparent based on genomic influences, it was of interest to determine whether there would be any RNP complex protein-specific contributions to regulation by VP30. Therefore, minigenomes from REBOV, ZEBOV and MARV were transcribed using each of the corresponding RNP complexes either with or without VP30. As a result of these experiments, it was apparent that transcription by EBOV RNP complexes was always regulated by the presence of VP30, even when a MARV genome

was used (Figure 32). Similarly, transcription by MARV RNP complexes was independent of regulation by VP30 even on EBOV templates. Thus, it appears that the dependence of EBOV transcription is not solely determined by the presence of genomic structures, such as hairpins, but that it is also a feature of the RNP complex itself. In contrast, the RNP complex seems inherently able to function without VP30. In order to determine if an individual component of the RNP complex was responsible for this altered phenotype, individual MARV proteins were substituted for their counterparts in the ZEBOV minigenome system in the absence of VP30 to determine if their presence could, at least partly, restore transcription. Perhaps unsurprisingly, no individual protein that could compensate for the absence of VP30 could be identified (Figure 32). This may indicate that the VP30-independent transcription phenotype onserved in MARV is somehow related to the overall structure of the RNP complex, with changes resulting in subtle alternations in protein-protein or protein-RNA interactions within the complex, rather than a substantial change in the function of any particular protein. Alternatively, this finding may be the result of recently reported specificities of protein-protein interactions within the RNP complex (Boehmann et al., 2005). In particular, it was demonstrated that VP35 and L proteins, while capable of physical interaction, as we could demonstrate upon coexpression by IFA (Figure 22), did not produce a functional complex (Boehmann et al., 2005). Thus it is possible that if either of these proteins is responsible for overcoming regulation by VP30, it will be necessary to substitute them in a pairwise fashion. It is also likely that additional restrictions on heterologous protein-protein interaction may exist when substitutions are made between MARV and EBOV, since these viruses are much less closely related than are the species of EBOV.

Recently, our understanding of what role VP30 might be playing in the regulation of transcription and replication has been further confounded by findings based on the MARV infectious clone system, which show that VP30 is required for the rescue of MARV, however, it does not require the Zn-binding motif previously shown to be required for transcriptional activation in EBOV (Enterlein et al., 2006). This suggests that MARV VP30 may play some kind of structural role in this context, perhaps by stabilizing the RNP complex, although why this would not also be the case in the minigenome system is unclear. This opens up an additional possibility regarding the lack of regulation of transcription by VP30. In light of these recent findings it may also be possible that the increased length of the hairpin in MARV (Figure 31) is in fact sufficient to overcome the need for the anti-terminator activity of VP30 and that subtle differences in the stability of protein-protein interactions within the RNP complex, or protein-RNA interactions result in a structural requirement for VP30 under some situations. However, the principles that would govern such a role are by no means clear from the existing data.

## 4.8 Inhibition of Filovirus Infection using Plasmid Expressed shRNAs

In addition to the well-documented application of minigenomes systems to the study of transcription and replication, we were interested to explore their more novel application to the screening of antiviral compounds. In particular, this work focuses on the identification of siRNA molecules for the inhibition of REBOV or ZEBOV infection. In order to first validate the use of minigenome systems in this type of application, we identified shRNA targets within the REBOV minigenome that were able to decrease reporter activity (Figure 33). Further analysis of these constructs in the cell culture

model of infection showed that they were also able to decrease vRNA production in infected cells, an indication of virus transcription/replication (Figure 33). Having confirmed that this approach was viable we similarly identified shRNA against the ZEBOV minigenome that were capable of inhibiting reporter accumulation in this system. Based on the availability of a GFP-expressing ZEBOV, further attempts to demonstrate the ability of this shRNA to control virus infection in cell culture were based on FACS analysis for GFP expression (Figure 35). Initial experiments carried out using lipid-mediated transfection in 293T cells demonstrated that these cells are not sufficiently susceptible to infection with GFP-ZEBOV to be used in this respect. Therefore, further attempts were made to test shRNA function using lipid-mediated transfection in Vero E6 cells. However, under these conditions a lack of efficacy was seen, in fact at high input doses of siRNA an apparent increase in GFP transcription was observed. While these results were generally interpreted as indicating insufficient transfection efficiency into the Vero E6 cells by lipid mediated transfection, an alternative explanation is that this increased GFP expression could itself be an indication of shRNA function when viewed in light of virus biology. Since this shRNA was designed to target the NP untranslated region, its most likely mode of action is to mediate degradation of NP mRNAs, although degradation of cRNA genome copies could arguably also occur. Thus the result of effective siRNA treatment would be a reduction of NP levels. However, if treatment is only modestly effective the levels of NP might be reduced to the lower end of the physiologically relevant levels of NP during virus infection, conditions which, according to current theories on the regulation of transcription and replication for *Mononegavirales*, would suggest an enhancement of transcription, albeit at the expense of replication.

When transient shRNA delivery was mediated by electroporation decreases in GFP expression could finally be observed (Figure 35). However, these decreases were still relatively small, and considering the apparent importance of efficient delivery for detectable shRNA activity, stable shRNA expressing cell lines were generated. Based on these cell lines larger decreases in GFP expression could be observed (Figure 37) consistent with the decreases in NP levels and reduced CPE following infection in these cell lines (Figure 38). Although, interestingly, by day 4 post-infection with a high MOI of virus, the percentage of GFP expressing cells was the same in either wild type or shRNA-expressing VeroE6 cells. However, from the intensity of GFP expression in the wild-type cells, which shows a pronounced decrease at this time point (Figure 37), it is clear that these cells are succumbing to infection, unlike their counterparts expressing the shRNA. Further experiments looking at the release of progeny virions from ZEBOV-GFP infected stable shRNA expressing cell lines showed decreases of between 1-1.5 logs after 3 or 4 days of infection. However, similar experiments using REBOV infection did not show any decrease in virus output (Figure 39). This suggests that the reduction in ZEBOV growth was due to a specific RNA interference mechanism and not to non-specific immune activation by the shRNA expression or plasmid delivery, nor was it likely to be due to defects in the cell lines themselves, since defects affecting the ability of the cell line to support ZEBOV growth would also be expected to impair growth of the closely related REBOV. These data also confirm that screening of compounds with antiviral activity against transcription and replication using the minigenome can be used for the identification of molecules that are similarly able to inhibit virus growth in tissue culture.

Finally, based on these data it was of interest to observe whether the decreases in virus growth mediated by shRNA expression would be sufficient to alter clinical outcome in the mouse model of ZEBOV infection. Based on the number of animals that lost more than 5 or 10% of their body weight during the course of infection it was apparent that ZEBOV shRNA plasmid treatment alone was insufficient to prevent weight loss in all animals in the challenge group from illness (Figure 40). However, if the plasmid was delivered prior to or shortly after infection weight loss was prevented in some of the animals (Figure 40). In contrast, the delivery of a scrambled shRNA plasmid together with mannose conjugated PEI, for macrophage and dendritic cell targeting, could prevent weight loss in some animals, while delivery together with galactose conjugated PEI, for liver targeting, prevented weight loss in all animals (Figure 40). This protection by the scrambled shRNA plasmid was not seen to be dependent on the time that treatment was initiated (Figure 40). Finally, when ZEBOV specific shRNA plasmids were delivered together with PEI they prevented weight loss regardless of the target cell type targeted or time of initiation of treatment (Figure 40).

While these data, on the whole, suggest that there is some specific protection associated with the ZEBOV shRNA not seen with either the scrambled shRNA-PEI complexes or the shRNA plasmid alone, it is clear that a more stringent infection model is required to clearly demonstrate this. In particular it is apparent that due to the low numbers of particles associated with a 10 $LD_{50}$ challenge model (~1 pfu (Bray et al., 1999) there is sufficient variation in the actual dose received by any given animal to confound analyses, particularly with such limited group sizes. This is seen when the untreated control group is observed. Here only two animals became ill, which

subsequently succumbed to infection, while in the ZEBOV shRNA treated group without PEI 4/5 animal became ill (Figure 40). It is, therefore, clear that further testing in 100 $LD_{50}$ and 1000 $LD_{50}$ models of ZEBOV infection, both of which are uniformly lethal in Balb/c mice (Bray et al., 1999), are required to more accurately gauge any therapeutic effects of shRNA treatment. As well it is anticipated that the use of these more stringent models will improve out ability to discriminate between specific and non-specific protection.

## 4.9 Summary

There has been a major increase in interest in filoviruses over recent years, both within the scientific community, as well as among the public. This has been has been due at least in part to a dramatic increase in the number of outbreaks in the last 10 years, as well as growing concern over the potential of these viruses to be used as bioweapons (Borio et al., 2002, Bray, 2003). Certainly their unusual degree of virulence has made them a popular subject of study for those wishing to investigate mechanisms underlying viral pathogenesis. In particular, the existence of closely related low and high virulence EBOV species provide a valuable opportunity to study the molecular determinants that give rise to the extremely virulent phenotype associated with ZEBOV. This potential is only just beginning to be fully realized with the development of reverse genetics for these viruses (Boehmann et al., 2005, Enterlein et al., 2006, Hoenen et al., 2006, Muhlberger et al., 1998, Muhlberger et al., 1999, Neumann et al., 2002a, Volchkov et al., 2001, Watanabe et al., 2004), which finally provide a context for the impact of individual

elements to be studies with respect to their effects on transcription/replication, morphogenesis/budding or even in the context of the complete virus life cycle.

This study has taken steps towards developing such reverse genetics tools for REBOV to both complement existing systems for ZEBOV and MARV and as independent tools in the study of this unique filovirus. By developing a novel Pol I minigenome system for REBOV we have demonstrated not only that this alternative approach to transcription can be successfully applied to cytoplasmically replicating members of *Mononegavirales*, but also that it compares favourably to the classical T7-driven approach. Further, using this system we could make a number of basic observations about transcription and replication in REBOV, including identifying the minimal protein components required for REBOV transcription/replication.

In addition, the application of the REBOV minigenome system, together with existing tools for other filoviruses, has implicated the efficiency of transcription/replication by the viral replicase complex as a potential determinant for pathogenesis, a finding consistent with the delayed growth of this virus in both cell culture and non-human primates (Fisher-Hoch et al., 1992, Jahrling et al., 1996). As well, analysis of these systems has suggested a contribution of the RNP complex proteins in determining the VP30-dependence of filovirus transcription. This is in contrast to a previously suggested model of regulation based strictly on the presence of genomic hairpins (Weik et al., 2002), which does not seem to account for the VP30 independent transcription observed in MARV.

Certainly, in addition to the need for insight in viral pathogenesis, the need to develop effective therapeutic and prophylactic measures against filovirus infection has

increased, along with their increasing occurrence. Therefore, we have applied filovirus minigenome systems as screening tools for the identification of novel inhibitors of transcription/replication and, in particular identified both REBOV and ZEBOV shRNAs that are capable of controlling infection in cell culture. In addition, preliminary data indicate that the ZEBOV shRNA may also reduce weight loss in a mouse model of ZEBOV infection, although further testing in a more stringent lethal model will be required in future to clearly demonstrate any therapeutic potential.

## 4.10 Future Studies

In addition to providing answers to some enduring questions regarding filovirus transcription and replication, this study has generated a number of new and intriguing ones that remain to be answered in the future. In many cases the answers to these questions will come through further applications of the reverse genetics systems that we have developed here or those that can now be developed based on the foundation of the minigenome system.

One deficiency that has become apparent based on attempts to assign function to specific genomic changes is that much of what is believed regarding filovirus transcription and replication is in fact drawn from analogy to VSV, but has never been experimentally confirmed. While it is likely that many of these assumptions will prove to be fundamentally valid, differences between these viruses no doubt exist. In particular it is unclear how the unusually long intergenic regions between many of the filovirus genes might affect downstream reinitiation of the replicase. These questions could be readily addressed through the use of bicistronic minigenomes containing the various viral

intergenic sequences. Similarly, the effect of gene overlaps and altered or duplicated transcriptional start signals on gene regulation can only be speculated at present.

Another area that has been increasingly complex in the last few years is in defining the role of VP30 in transcription and replication. While our data, together with that of others (Enterlein et al., 2006), imply a possible structural role for VP30 in RNP formation under some circumstances, the identification of a predicted hairpin structure in MARV analogous to the one in ZEBOV that has been linked to regulation of transcription by VP30, requires that the influence of this structure is ruled out. This could perhaps be most directly addressed by exchanging the hairpins of ZEBOV and MARV in the minigenome system. Alternatively, ZEBOV minigenome mutants could be constructed in which the existing hairpin is expanded to mimic that of MARV through the incorporation of additional complementary nucleotides.

Certainly, an immediate goal for the continuation of the research is further testing of ZEBOV-specific shRNAs in more stringent models of ZEBOV infection. If warranted, further testing could be undertaken to establish appropriate optimal delivery routes and dosing regimens, as well as to explore alternative delivery vehicles. In particular, the use of an infectious delivery vehicle, such as a recombinant adenovirus might be necessary to achieve widespread delivery of the shRNA to target tissues in larger animal models. Although our current focus is on the application of RNA interference as a therapeutic approach, this screening platform could also be used as a first screen to evaluate any number of other antiviral approaches and could ideally be extended to include drug library screening, through collaboration with the appropriately equipped institutions. In addition to this therapeutic role, the ability to target viral

proteins through RNA interference-based strategies will likely be valueble in facilitating future attempts to study the roles of specific proteins in pathogenesis. In addition to allowing protein knock-down experiments where the loss of function can be analyzed, they will also allow studies of the capacity of mutant proteins to complement loss of function due to wild-type protein inhibition. This may be particularly important for confirming negative rescue results with the infectious clone system that are due to mutations result in a low-viability of progeny. Currently it is not possible to eliminate technical failures in these cases and so as a confirmation of such findings would have substantial value to reverse genetics.

Finally, perhaps one of the most significant extensions of this work, with respect to the study of filovirus pathogenesis, will be the application of the tools and knowledge gained through this study to the development of future reverse genetics tools for REBOV. Indeed, based on precedent from the ZEBOV system, the available REBOV minigenome system could be relatively easily extended to an iVLP system by the additional cotransfection of VP40 and GP. This would provide a system to study the packaging signals of REBOV in further detail under BSL-2 conditions. Additionally, the information acquired through the optimization of the REBOV minigenome can now be applied to assist in the development of an infectious clone system for this important virus. In particular, information about optimal protein ratios and the promoter sequences that provide the best balance of efficient T7 transcription and subsequent viral transcription and replication will likely be very important for the success of this system. This is particularly the case since reported titres from the ZEBOV infectious clone systems have been very low ($\sim 10^3$) and, in comparison to ZEBOV, REBOV is compromised in its

tissue culture growth, an observation substantiated by the reduced transcription and replication by the REBOV RNP complex shown in this study. However, while the establishment of a REBOV infectious clone system will likely require careful optimization with respect to these parameters in order to obtain a successful system for virus rescue, its value to the study of filovirus pathogenesis would be considerable. As such we are currently undertaking to generate such a system based on the existing tools described here. With such a system for the generation of infectious virus entirely from cloned cDNA, the implications of the genetic changes in REBOV, identified through our sequencing study can be directly evaluated.

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## Appendix A - Primers

**Table 1. Diagnostic primer pairs used for initial sequencing of REBOV.**

| Primer Name | Target Gene | Primer Sequence |
|---|---|---|
| NPf | NP (nt 661-1090) | 5'- CCAAGAAAATGCCGACAGCTTCC |
| NPr | | 5'- CAAGAAATTAGTCCTCATCAATC |
| GPf | GP (nt 7665-7996) | 5'- GCATCTACATTGAGGGTGTAA |
| GPr | | 5'- CCATTGTCTCCAACCTGTCCA |
| Lf | L (nt 15160-1555) | 5'- CTATCAAGCCGCGATGCCCTTC |
| Lr | | 5'- CAGAGGTGTTTGTATTCCGGAATC |

**Table 2. Primers for pGEX expression of REBOV proteins and peptides for antibody production**

| Primer Name | Target Gene | Primer Sequence |
|---|---|---|
| NP (frag 1) F | NP (nt 702-910) | 5'- GACGAATTCCATCATGCTTACCAAGGTG AC |
| NP (frag 1) R | | 5'- GACGAATTCTTGCCCTGCATTTGCTTCTG TAGTCTCC |
| NP (frag 2) F | NP (nt 1931-2485) | 5'- GACGAATTCGATCATGAGGATGACAATA AAGC |
| NP (frag 2) R | | 5'- GACGAATTCCTCATCCTTCATCATGTG |
| VP35 (ORF) F | VP35 ORF | 5'- TTGAATTCACCTTAGGACCATTGTCAAG AGG |
| VP35 (ORF) R | | 5'- TTGAATTCTTAGATCTTAAGTCC |
| VP30 (ORF) F | VP30 ORF | 5'- GACGAATTCCCGGATGATGGAGCATTCA AGAGAACGG |
| VP30 (ORF) R | | 5'- GACCTCGAGTTCTATTCAACTGTACTTGA CC |
| L (frag1) F | L (nt 11550-11717) | 5'- AATGAATTCATGGCTACCCAGCATACCC AG |
| L (frag1) R | | 5'- AATGAATTCGTCGAACTTAAGTCGATAT ATATG |
| L (frag2) F | L (nt 16476-16823) | 5'- AATGAATTCTGGAGAAGTAGGAAATCTC GG |
| L (frag2) R | | 5'- AATGAATTCTCGGTGAGGACTTGATACA TC |

* Restriction sites are shown underlined

**Table 3. Primers for pCAGGS express plasmids**

| Primer Name | Target Gene | Primer Sequence |
|---|---|---|
| NP(ORF) F | NP (ORF) | 5'- GCGGAATTCGTCGATATGGATCGTG |
| NP(ORF) R | | 5'- GCGGAATTCCGGTCAAGTCTTAAGTG |
| VP35(ORF)F | VP35 ORF | 5'- TTGAATTCACCTTAGGACCATTGTCAAGAGG |
| VP35(ORF)R | | 5'- TTGAATTCTTAGATCTTAAGTCC |
| VP30 (ORF)F | VP30 ORF | 5'- GACGAATTCCCGGATGATGGAGCATTCAAGAGAACGG |
| VP30(ORF)R | | 5'- GACCTCGAGTTCTATTCAACTGTACTTGACC |
| VP40 (ORF) F | VP40 ORF | 5'- GACGAATTCAAAATGAGGCGCGGAGTGTTACC |
| VP40 (ORF) R | | 5'- GACCTCGAGTTACTAGCAGAATAATAGCTCG |
| L(11550)F | L ORF (nt 11550-14860) | 5'- AATGGTCTCGAATTCCACCATGGCTACCCAGCATACCCAGTACC |
| L(15000)R | | 5'- AATGCTAGCAATTGGTCACAATG |
| L(15000)F | L ORF (nt 15000-18188) | 5'- AATGCTAGCAGATGCTCTACAGAAAATTAG |
| L(18188)R | | 5'- AATAGATCTCTATGTGTGATTGGAATATATTAACCC |

* Restriction sites are shown underlined

**Table 4. Primers for REBOV CAT and GFP reporter minigenome construction.**

| Primer Name | Target | Primer Sequence |
|---|---|---|
| Leader F | Leader (nt 1-463) | 5'- AATGAAGACGGATCGACTTCCCCTTGTTCGAC |
| Leader R | | 5'- AATGAAGACGGGGGCGGACACACAAAAAGAAAAAAGG |
| CAT F | CAT ORF | 5'- AATGGTCTCGTATTCCGTCTTCCACAGAAGACGGGTACTTACGCCCCGCCCTGCC ACTCATCGC |
| CAT R | | 5'- AATGGTCTCGCGATATGGAGAAAAAAATCACTGG |
| GFP F | eGFP ORF | 5'- AATGGTCTCGTATTCCGTCTTCCACAGAAGACGGGTACTTAGTGATGGTGATGGTG ATGGGATCC |
| GFP R | | 5'- AATCGTCTCGCGATATGGTGAGCAAGGGCGAGGAGC |

| | | |
|---|---|---|
| Trailer F | Trailer (nt 18189-18891) | 5'- AATGAAGACGGTATTGGACACACAAAAAGGA AAAAATTGG |
| Trailer R | | 5'- AATGAAGACGGGTACTAAATCATCATAGTAT GAGG |

* Restriction sites are shown underlined

**Table 5. Primers for construction of 1G-3G T7-driven REBOV minigenomes.**

| Primer Name | Target | Primer Sequence |
|---|---|---|
| del_fw_1 | Leader (nt 1-20) | 5'- Phos-CGGACACACAAAAAGGAAAAA |
| del_rev_1 (3G) | T7 promoter | 5'- Phos-**CCC**TATAGTGAGTCGTATTAC |
| del_rev_2 (2G) | | 5'- Phos-**CC**TATAGTGAGTCGTATTACA |
| del_rev_3 (1G) | | 5'- Phos-**C**TATAGTGAGTCGTATTACAA |

* Inserted residues are shown in bold

# Appendix B - Reagents

### Coomassie brilliant blue R-250 stain

| | |
|---|---|
| Coomassie brilliant blue R250 | 0.25% (w/v) |
| Glacial acetic acid | 10% (v/v) |
| Methanol | 45% (v/v) |
| Distilled water | 45% (v/v) |

Dissolve the dye in methanol and let stand at room temperature for 3 h. Add acetic acid and water. Stir

### Coomassie destain

| | |
|---|---|
| Glacial acetic acid | 10% (v/v) |
| Methanol | 45% (v/v) |
| Distilled water | 45% (v/v) |

Add acetic acid and the methanol to the water. Mix

### 6x Nucleic Acid Gel Loading Buffer

| | |
|---|---|
| Bromphenol blue | 0.25% (w/v) |
| Sucrose | 40% (w/v) |

Dissolve the dye and sucrose in sterile distilled water. Store finished solution at 4°C to avoid mould growth

### 4x SDS-PAGE Gel Loading Buffer

| | |
|---|---|
| Tris-HCl (pH 6.8) | 240 mM |
| β-mercaptoethanol | 20% (v/v) |
| Sodium dodecyl sulphate | 8.0% (v/v) |
| Glycerol | 40% (v/v) |
| Bromphenol blue | 0.2% (w/v) |

Dissolve contents in the required volume sterile distilled water. If β-mercaptoethanol is added, store at -20°C in 1mL aliquots. Alternatively, add all reagents except β-mercaptoethanol and store at RT. Add β-mercaptoethanol immediately prior to use.

**50X Tris-Acetate EDTA Buffer**

| | |
|---|---|
| Tris HCl | 2.0 M |
| Acetic acid | 1.0 M |
| EDTA | 50 mM |

Dissolve reagents in sterile distilled water. Dilute to 1x with $sdH_2o$ prior to use.

**10x Tris-Glycine Buffer**

| | |
|---|---|
| Tris HCl | 250 mM |
| Glycine | 1.9 M |
| Sodium dodecyl sulphate | 1.0 % (w/v) |

Dissolve reagents in sterile distilled water. Dilute to 1x with $sdH_2o$ prior to use.

**Pwo PCR buffer (10× conc.)**

| | |
|---|---|
| Tris-HCl | 100 mM |
| KCl | 250 mM |
| $(NH_4)_2SO_4$ | 50 mM |
| $MgSO_4$ | 20 mM |

Dissolve Tris HCl in sterile distilled water. Dissolve remaining components and stir. Adjust pH to 8.85 at 20°C.

**Buffer P1 (Resuspension Buffer)**

| | |
|---|---|
| Tris-HCl | 50 mM |
| EDTA | 10 mM |
| RNase A | 100 µg/µl |

Dissolve Tris-HCl and EDTA in 80% of the final volume of sterile distilled water. Adjust pH to 8.0 with HCl. Adjust the volume to 100% with sterile distilled water. Add Rnase A before use. Following addition of RNAse A store at 4-8°C

**Buffer P2 (Lysis Buffer)**

| | |
|---|---|
| NaOH | 200 mM |
| Sodium dodecyl sulphate | 1% (w/v) |

Dissolve NaOH in sterile distilled water. Add SDS and adjust to final volume. Store at RT

**Buffer P3 (Neutralization Buffer)**

| | |
|---|---|
| Potassium acetate | 1 M |

Dissolve potassium acetate in 50% final volume sterile distilled water. Adjust pH to 5.0 with glacial acetic acid. Adjust to final volume and store at RT.

**SOC Medium**

| | |
|---|---|
| Tryptone | 2% (w/v) |
| Yeast Extract | 0.5% (w/v) |
| NaCl | 10 mM |
| $MgSO_4$ | 10 mM |
| $MgCl_2$ | 10 mM |

Dissolve ingredients in sterile distilled water. Dispense into 25mL aliquots in glass bottles and sterilize by autoclaving at 121°C for 30 minutes. Store at RT.

**Tris-Glycine Transfer Buffer**

| | |
|---|---|
| Trisma Base | 0.3% (w/v) |
| Glycine | 1.4% (w/v) |
| Methanol | 20% (v/v) |

Dissolve ingredients in distilled water and store at RT.

# Appendix C - *Reston ebolavirus* (strain Pennsylvania) Genome Sequence

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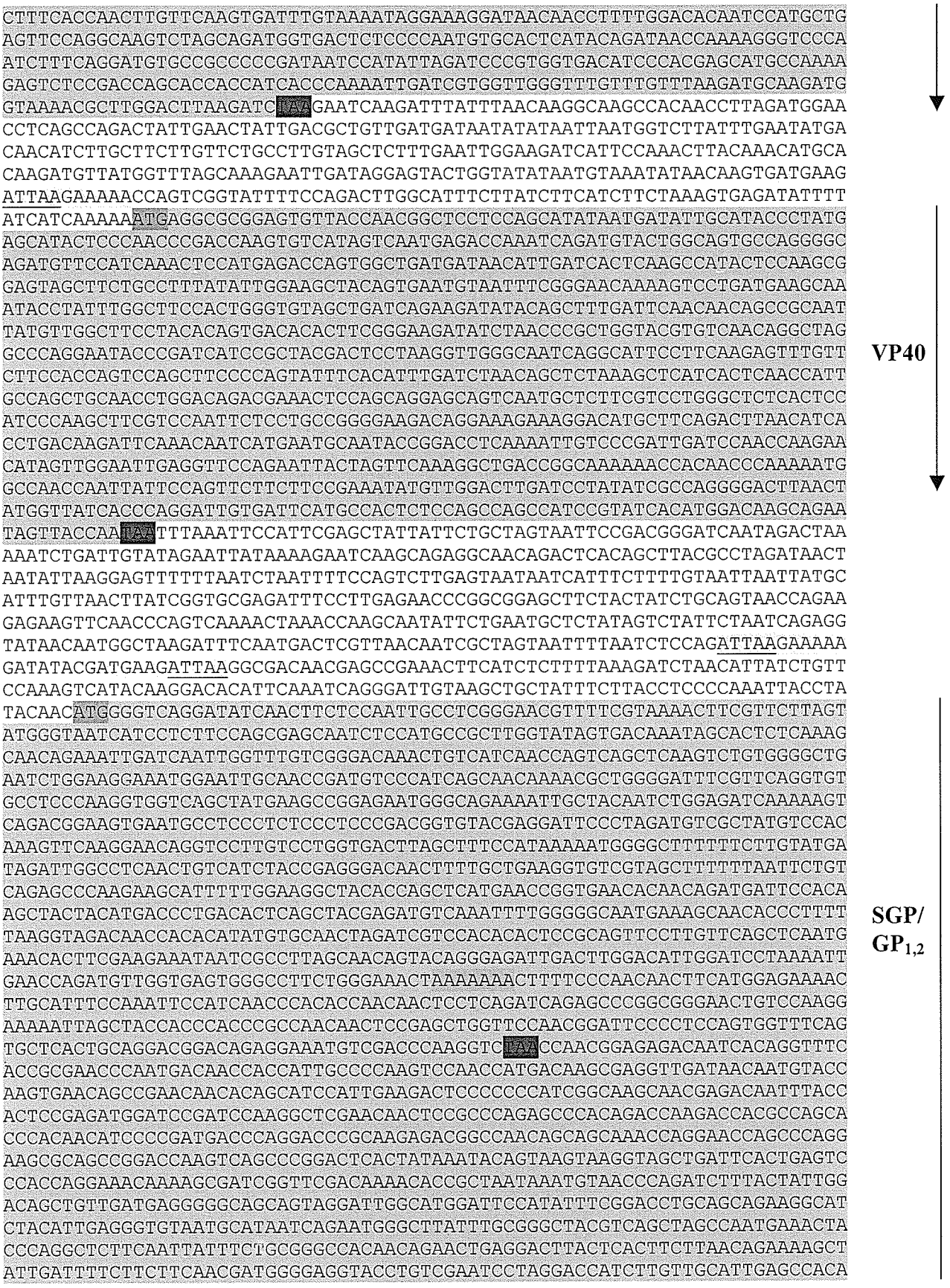
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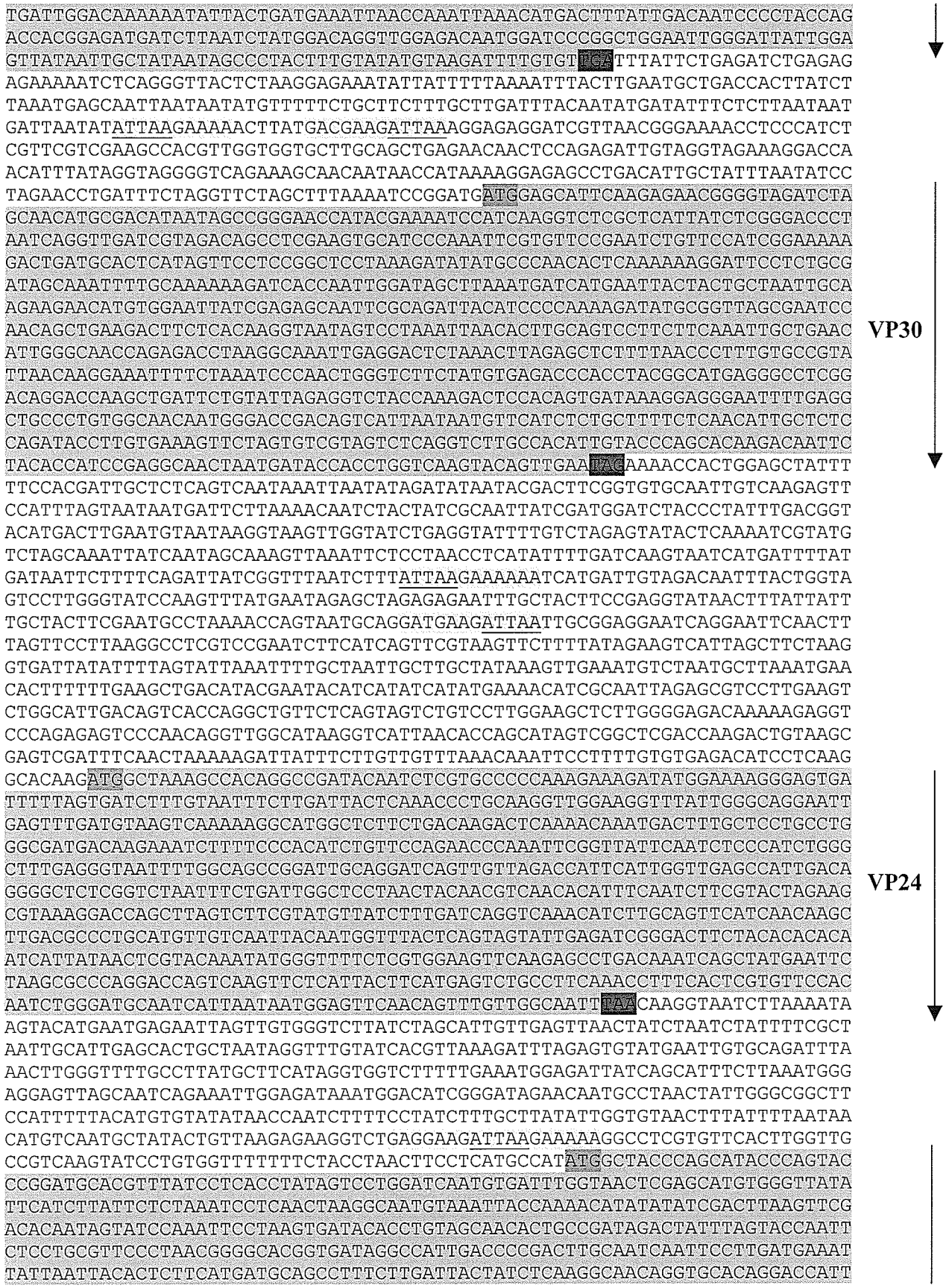
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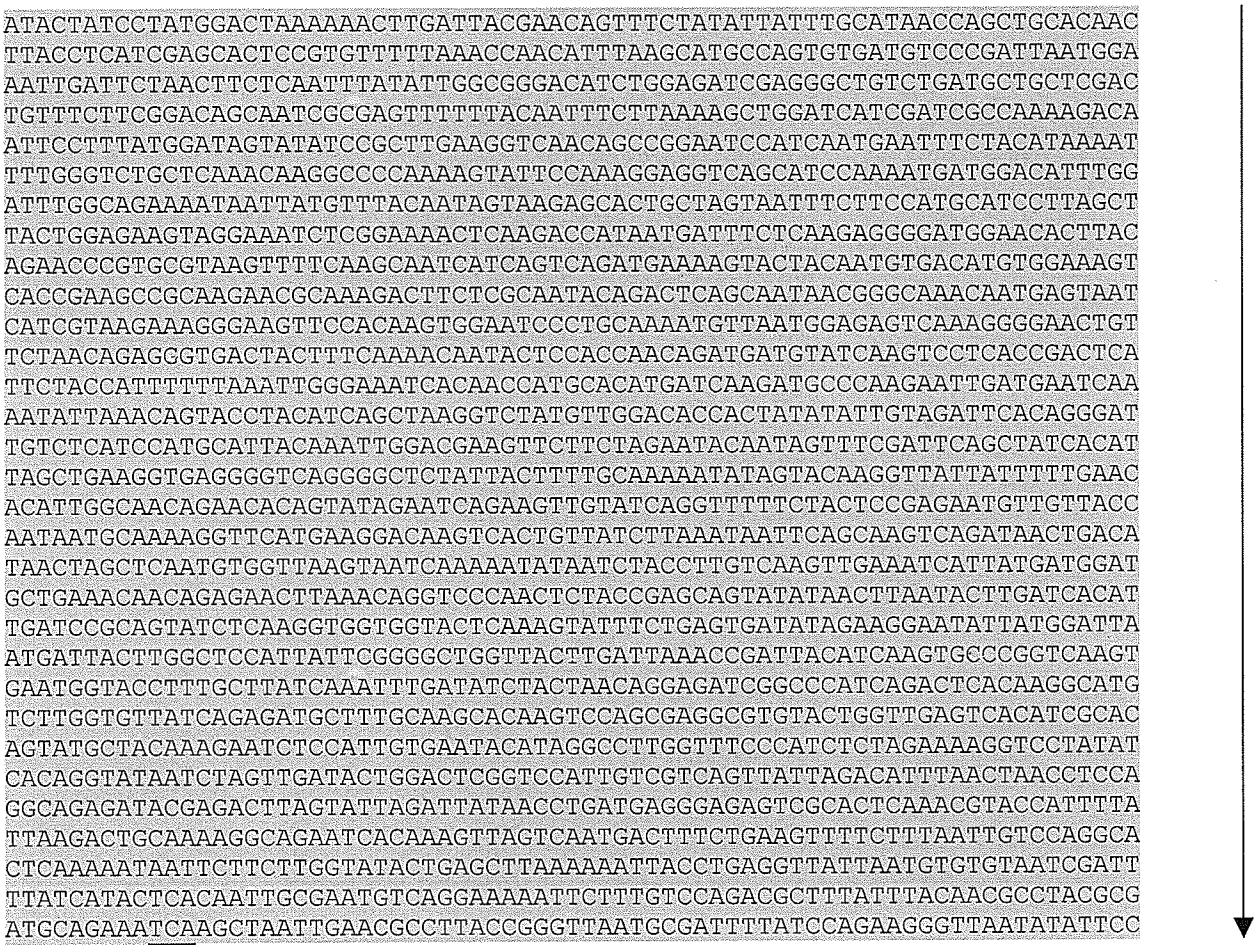


**Legend**

- Open Reading Frame (ORF)
- Transcriptional Start/Stop signals
- Translational Start
- Translational Stop
- Glycoprotein RNA editing site

## Appendix D - List of Abbreviations

BME - β-mercaptoethanol
BSL2 - Biosafety level 2
BSL4 – Biosafety level 4
CAT – Chloramphenicol acetyl transferase
cDNA - Complementary DNA
CFA – Complete Freund's adjuvant
CMC – Carboxymethyl cellulose
cRNA - Complementary RNA
DC – Dendritic cell
DIC – Disseminated intravascular coagulation
DMEM – Dulbecco's modified Eagle's medium
DMSO – Dimethyl sulphoxide
DNA - Deoxyribonucleic acid
dsRNA – Double stranded RNA
EBOV – Ebola virus
EC – Endothelial cell
EDTA – Ethylenediamine tetra-acetic acid
EHF - Ebola haemorrhagic fever
EMEM – Eagle's minimal essential medium
EtBr – Ethidium Bromide
FACS – Fluorescence-activated cell sorting
FFU - Focus-forming unit
GFP – Green fluorescent protein
GP - Glycoprotein
GST – Glutathione-S-transferase
HDV – Hepatitis delta virus
ICEBOV – *Ivory Coast ebolavirus*
ICTV – International Committee on the taxonomy of viruses
IFA – Immunofluorescence assay
IFN - Interferon
i.m.- Intra-muscular
i.p. – Intra-peritoneal
IPTG - Isopropyl β-D-1-thiogalactopyranoside
iVLP – Infectious virus-like particle
jetPEI-Gal – Linear polyethylenamine conjugated to galactose
jetPEI-Man - Linear polyethylenamine conjugated to mannose
L – Viral RNA-dependent RNA polymerase
LB - Lauria-Lenox broth
Luc - Luciferase
MARV – *Lake Victoria marburgvirus*
MCS – Multiple cloning site
MOI - Multiplicity of infection
mRNA - Messenger RNA
MVA-T7 - Modified vaccinia virus Ankara

MVB - Multivesicular body
N - Nucleocapsid protein
NCR - Non-coding region
NNS – Non-segmented, negative sense, single-stranded
NP - Nucleoprotein
ORF – Open reading frame
P - Polymerase cofactor protein/Phosphoprotein
PCR - Polymerase chain reaction
PEG – Polyethylene glycol
PEI - Polyethylenimine
PFA - Paraformaldehyde
PKR - RNA-activated protein kinase
PNK – Polynucleotide kinase
Pol I – RNA polymerse I
PTGS – Post-transcriptional gene silencing
PVDF – Polyvinylidine difluoride
RdRp - RNA-dependent RNA polymerase
REBOV - *Reston ebolavirus*
RISC – RNA-induced silencing complex
RNA - Ribonucleic acid
RNase - Ribonuclease
RNAi – RNA interference
RNP – Ribonucleoprotein
RT-PCR – Reverse transcriptase polymerase chain reaction
SDM – Site-directed mutagenesis
SDS – Sodium dodecyl sulphate
SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEBOV – *Sudan ebolavirus*
siRNA – Small interfering RNA
shRNA – Small hairpin RNA
UTR - Untranslated region
UV - Ultraviolet
T7 – Bacteriophage T7 RNA polymerase
TF - Tissue factor
TLR – Toll-like receptor
TM - Transmembrane
Tris - Tris(hydroxymethyl)-aminomethane
TSS – Transformation and storage solution
VHF – Viral haemorrhagic fever
VLP – Virus-like particle
VP – Virion protein
vRNA – Viral RNA
VSV – Vesicular stomatitis virus
vTF7-3 - Recombinant vaccinia virus expressing the T7 RNA polymerase
ZEBOV – *Zaire ebolavirus*