Establishment and characterization of plasmid-driven minigenome rescue systems for Nipah virus: RNA Polymerase I- and T7-catalyzed generation of functional paramyxoviral RNA

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Abstract

In this study we report the development and optimization of two minigenome rescue systems for Nipah virus, a member of the *Paramyxoviridae* family. One is mediated by the T7 RNA polymerase supplied either by a constitutively expressing cell line or by transfection of expression plasmids, and is thus independent from infection with a helper virus. The other approach is based on RNA polymerase I-driven transcription, a unique approach for paramyxovirus reverse genetics technology. Minigenome rescue was evaluated by reporter gene activities of (*i*) the two different minigenome transcription systems, (*ii*) genomic versus antigenomic-oriented minigenomes, (*iii*) different ratios of the viral protein expression plasmids, and (*iv*) time course experiments. The high efficiency and reliability of the established systems allowed for down-scaling to 96-well plates. This served as a basis for the development of a high throughput screening system for potential antivirals that target replication and transcription of Nipah virus without the need of high bio-containment. Using this system we were able to identify two compounds that reduced minigenome activity.

Keywords

Paramyxovirus; Nipah virus; RNA polymerase I-driven minigenome rescue; antiviral drug screening; high throughput

Introduction

Nipah virus (NiV), together with the closely related Hendra virus (HeV), are recently emerged deadly viruses belonging to the family *Paramyxoviridae*. Their genetic constitution, high virulence and wide host range set them apart from other paramyxoviruses. NiV and HeV have been proposed to form a new genus, *Henipavirus*, within the subfamily *Paramyxovirinae* and are the only zoonotic paramyxoviruses with high lethality (Chua et al., 2000a; Harcourt et al., 2000). NiV was first identified in Malaysia in 1998/99 (Chua et al., 1999; Chua et al., 2000b; Goh et al., 2000) as the causative agent of an outbreak of respiratory illness in pigs and both

encephalitis and massive cerebral hemorrhages in humans (Parashar et al., 2000). Subsequently in 2001-2005, NiV outbreaks occurred in Australia, Malaysia, Singapore, India, and Bangladesh (Butler, 2004; Chadha et al., 2006; Eaton et al., 2006; Enserink, 2004; Hsu et al., 2004; WHO, 2004). NiV infections can result in a 40 to 70% mortality rate, primarily from fatal encephalitis, and there is increasing evidence of person-to-person transmissions (Hsu et al., 2004; WHO, 2004). NiV is one of several newly emerging and reemerging viruses, classified as a biosaftey level 4 (BSL-4) pathogen and is designated a Category C Priority Pathogen in the NIAID Biodefense Research Agenda. It has been cited as a potential agent of bioterrorism due to its high virulence, evidence of person-to-person transmission, and the absence of vaccines or chemotherapeutics for this virus (Lam and Chua, 2002). In addition to causing acute disease, 3 to 7% of infected patients exhibit a late onset or relapsed encephalitis months to years after the initial infection (Tan et al., 2002), thereby increasing the risk of community exposure.

As a member of the *Paramyxoviridae* family, NiV contains a negative stranded, nonsegmented RNA genome, with a gene arrangement similar to that of other family members: 3′– nucleocapsid protein (N) – phosphoprotein (P)/non-structural proteins V, W and C – matrixprotein (M) – fusionprotein (F) – glycoprotein (G) – RNA-dependent RNA polymerase (L) – 5′. The genome length of 18,246 nucleotides (nt) is approximately 2,700 nt longer than others in the family, only surpassed in length by the recently discovered Beilong virus (Li et al., 2006). Translation of its six transcription units results in the synthesis of six major structural proteins and three non-structural proteins V, W and C from the P mRNA (Harcourt et al., 2000). Recently, it has been shown that three of the viral structural proteins, N, P, and L, are necessary and sufficient for both transcription and replication of a NiV minigenome (a reporter gene flanked by the 5′ and 3′ non-coding regions of NiV) (Halpin et al., 2004). Furthermore, this confirms that all *cis*-acting elements for initiating transcription and replication reside within the terminal genome sequences as shown for other *Mononegavirales* including Ebola and Marburg virus (Groseth et al., 2005; Mühlberger et al., 1998; Mühlberger et al., 1999), Respiratory syncytial virus (Peeples and Collins, 2000), Sendai virus (Calain and Roux, 1993), Borna disease virus (Rosario, Perez, and de la Torre, 2005; Schneider et al., 2003) and recently, NiV (Halpin et al., 2004). Thus, the NiV minigenome rescue system provides a powerful tool to study the processes of replication and transcription and can eliminate biosafety concerns associated with the use of infectious virus. The usefulness of similar systems have been demonstrated for other BSL-4 pathogens such as Zaire ebolavirus and Marburg virus (Mühlberger et al., 1998; Mühlberger et al., 1999; Weik et al., 2005), Reston ebolavirus (Boehmann et al., 2005; Groseth et al., 2005), Lassa fever virus (Hass et al., 2004), Rift Valley fever virus (Gauliard et al., 2006; Gerrard et al., 2007; Ikegami, Peters, and Makino, 2005; Lopez et al., 1995; Prehaud et al., 1997), Crimean Congo hemorrhagic fever virus (Flick et al., 2003b), and Nipah virus (Halpin et al., 2004; Yoneda et al., 2006). However, the majority of these systems were dependent on the T7 RNA polymerase (T7) that had to be supplied in *trans*. To avoid the necessity for an exogenous RNA polymerase for minigenome expression, the endogenous RNA polymerase I (Pol I) was used in this study to generate NiV minigenomes. Initial concerns about the use of an enzyme located in the nucleus to drive a reverse genetics system for viruses exclusively replicating in the cytoplasm were not justified. Export of Pol I transcripts from the nucleus to the cytoplasm seems not to be a limiting factor for minigenome rescue systems; we recently demonstrated this for several members of the family *Bunyaviridae* (Flick et al., 2003a; Flick et al., 2004; Flick, Elgh, and Pettersson, 2002; Flick et al., 2003b; Flick and Pettersson, 2001; Gauliard et al., 2006) and for the Reston ebolavirus (Groseth et al., 2005).

In summary, in this study we demonstrate for the first time the successful use of an RNA Pol I-driven minigenome rescue system, originally developed for influenza virus (Flick et al., 1996; Neumann, Zobel, and Hobom, 1994; Zobel, Neumann, and Hobom, 1993), for a

paramyxovirus. The concept of the present technology is based on the transfection of cells with the NiV minigenome system which results in the expression of CAT reporter gene whose level of enzymatic activity directly reflects the extent of viral replication. Such a helpervirus-free system driven by an endogenous enzyme has strong potential to be the basis for the establishment of an efficient and reliable screening system for potential antivirals and for the development of a reverse genetics system for members of the *Paramyxoviridae* family.

Results

Establishment of a helper virus-free T7 RNA polymerase-driven NiV minigenome rescue system

The previously published T7 RNA polymerase-driven minigenome rescue system for NiV was dependent on infection with the recombinant vaccinia virus MVA-T7 (Halpin et al., 2004). To eliminate the additional step of MVA-T7 infection as well as potential problems due to the introduced vaccinia virus proteins, we established a helpervirus-free T7 RNA polymerase-based approach by using a cell line stably expressing the T7 polymerase (BHK-T7 724-5cl; provided by Ilya Frolov, University of Texas Medical Branch, Galveston, TX) (Fig. 1A). Using this cell line and the previously described ratios for the viral polymerase L, the nucleoprotein N, and the phosphoprotein P, we performed minigenome rescue in 24-well plates resulting in reliable expression levels (Fig. 1B). To improve the overall signal-to-noise ratio, cells were transfected with different amounts of minigenome plasmids (0.05 to 0.4 μg) and cell lysates were assayed for CAT activity 24 h post-transfection (data not shown). An optimal signal-to-noise ratio was achieved with 0.2 μg of pT7 NiV-CAT (-) (NiV minigenome in genomic orientation) for a 24-well plate (Fig. 1B). In absence of the expression plasmids L, N, and P, higher amounts of transfected minigenome plasmids (i.e., > 0.3 μg) showed strong background of reporter gene expression levels indistinguishable from the rescued specific signals. Omitting only one of the expression plasmids (L or N or P) resulted also in high background signals (data not shown). The background activity observed in the presence of larger amounts of transfected minigenome plasmids likely results from the recognition of cryptic promoter elements within the reporter minigenome plasmid by the T7 polymerase and/or cellular polymerases.

For even more flexibility and not to be restricted to one specific cell line, a helpervirus-free, completely plasmid-based T7 NiV minigenome rescue technology was developed. To this end, the T7 RNA polymerase was provided by co-transfection of a plasmid-encoded T7 polymerase (Figs. 1A and C). Initially, two different eukaryotic T7 polymerase expression systems were used. One, pCAGGS/T7, is under the control of the chicken β–actin promotor (kindly provided by Yoshihiro Kawaoka, University of Wisconsin-Madison, USA) (Kobasa et al., 1997; Niwa, Yamamura, and Miyazaki, 1991) and the other, pCMV-T7, is under the control of the Cytomegalovirus (CMV) immediate early promoter and enhancer element (kindly provided by Michèle Bouloy, Institute Pasteur, France). Using human embryonic kidney (293T/17) cells, stronger pT7 NiV-CAT (-) reporter gene signals were observed with pCAGGS/T7 compared to the pCMV-T7 expression plasmids (Fig. 1C: lane 2 versus 4). This could be due to higher T7 expression levels in case of pCAGGS/T7 resulting in better expression of NiV support plasmids. In addition, this helpervirus-free minigenome rescue approach was also performed successfully with pCAGGS/T7 in human epithelial HeLa cells (Fig. 1C, lane 6) demonstrating that NiV minigenome rescue is possible without the use of MVA-T7 or the restricted use of T7 RNA polymerase stable expressing cell lines and can be used in a variety of mammalian cell lines. Based on the better signal intensity, we decided to use 293T/17 cells and the pCAGGS/T7 expression plasmid in all subsequent experiments.

Establishment of an RNA polymerase I-driven minigenome rescue system

Since a T7-driven minigenome system is still dependent on the exogenous polymerase to generate the NiV minigenome RNAs, we developed a system based on the cellular Pol I, a unique approach within the field of paramyxoviruses. For this, cDNAs of NiV minigenomes in genomic [vRNA, (-)] or antigenomic [cRNA, (+)] orientation that contained the CAT reporter gene flanked by the human Pol I promoter and the mouse Pol I terminator were designed. A Pol I-component-containing plasmid (pLD9, unpublished data, L. Deflubé and R. Flick) served as backbone to incorporate NiV minigenomes in genomic and antigenomic orientation [pPol I NiV-CAT (-) and pPol I NiV-CAT (+)] and was used in transfection experiments to deliver functional NiV minigenomes. CMV-driven expression plasmids for NiV structural proteins were generated from the previously described T7-dependent expression plasmids (Halpin et al., 2004) by inserting the ORF's into the pcDNA 3.1 (Invitrogen) backbone.

One substantial advantage of the Pol I- versus the more classical T7-driven system is the well defined transcription start and termination sites. Pol I transcription results in RNAs without any additional nucleotides on either end or other modifications (e.g., poly A tail, cap structure) (Flick and Hobom, 1999; Neumann, Zobel, and Hobom, 1994; Zobel, Neumann, and Hobom, 1993) and therefore there is no need to add ribozyme sequences or use run-off *in vitro* transcripts to generate (in subsequent, inefficient steps) the functional minigenome. After transfection of the Pol I-driven minigenome plasmids into eukaryotic cells (e.g., 293T/17), the cellular Pol I generates minigenome transcripts with authentic viral genome ends.

Comparison of T7- and Pol I-generated NiV minigenomes

In a first step, we tested our newly established Pol I-driven NiV minigenome rescue system in 293T/17 cells for functionality. Transfection of all plasmids resulted in the expression of CAT protein, as measured by its enzymatic activity (Fig. 1D, lane 2). The reporter gene expression levels were strong and showed no background signals (Fig. 1D, lane 1). After demonstrating the functionality of the Pol I-driven NiV minigenome rescue system, we directly compared the efficacies of both minigenome rescue systems in the same cell line. For this, 293T/17 cells were transfected with NiV minigenome plasmids [pT7 NiV-CAT (-) and pPol I NiV-CAT (-)] along with NiV L, N, and P protein-expressing plasmids, either under T7- or CMV-control, and pCAGGS/T7 (Fig. 1D). Negative controls, in which no NiV polymerase support plasmid had been transfected, are shown in the odd lanes. Taking into account that only half the amount of pPol I NiV-CAT (-) was transfected compared to pT7 NiV-CAT (-), the Pol I-driven minigenome system showed a strong reporter gene signal and the level of NiV minigenome expression is comparable with the T7-driven minigenome system (Fig. 1D: lanes 2 versus 4). Overall, both minigenome rescue systems exhibit comparable rescue efficiencies and CAT activities in the same cell line. This result indicates that the Pol I-driven minigenome system might be a useful alternative to analyze transcription and replication processes for paramyxoviruses and to serve as a control for antiviral drug screening assays.

Analysis and characterization of Pol I-driven minigenome replication

In the previously described experiments, minigenomes in genomic (vRNA) orientation were analyzed. In order to determine if NiV minigenome replication in addition to transcription occurs, minigenomes in cRNA orientation were used. The antigenomic RNA transcripts need to first undergo a replication step (cRNA → vRNA) in order to generate a functional template for minigenome transcription (vRNA → mRNA). Hence, CAT reporter gene expression in such experiments is dependent on replication and transcription.

Relative minigenome expression levels of genomic [pPol I NiV-CAT (-)] and antigenomic [pPol I NiV-CAT (+)] minigenomes in 293T/17 cells using various amounts of transfected

plasmid DNAs were determined (Fig. 2). For this, cells were transfected in 24-well plates and harvested 48 h later for CAT analysis. Reporter gene expression was observed from both genomic (Fig. 2A) and antigenomic (Fig. 2B) constructs. As little as 0.05 μg of pPol I NiV-CAT (-) (Fig. 2A, lane 4) and 0.025 μg of pPol I NiV-CAT (+) (Fig. 2B, lane 2) were sufficient to produce detectable levels of CAT activity. In the absence of NiV support plasmids, only residual CAT background activity was observed (Figs. 2A and B: odd lanes). The antigenomic minigenome pPol I NiV-CAT (+) resulted in slightly stronger reporter gene activities compared to the minigenome in genomic orientation pPol I NiV-CAT (-), even at lower amounts of transfected plasmid. We therefore decided to use this antigenomic-oriented minigenome in subsequent experiments.

We sought to further characterize the newly established Pol I-driven NiV minigenome rescue system by performing time course experiments to determine time points of maximum reporter gene expression. To this end, 293T/17 cells were transfected with optimal amounts of plasmids which resulted in optimal signal-to-noise ratios (pPol I NiV-CAT (+): 1.0 μg, NiV L: 0.2 μg, NiV N: 0.6 μg, NiV P: 0.2 μg) and harvested at different time points between 10 h and 86 h post-transfection (Fig. 2C). CAT activity was first detected at 24 h (Fig. 2C, lane 6) with a significant increase in signal between 27 and 40 h post-transfection (Fig. 2C: lanes 8 and 10) and reached a plateau after 52 h (Fig. 2C: lanes 14, 16, 18). We decided to harvest cells in all subsequent experiments 48 h post-transfection. It is noteworthy that even after 86 h post-transfection, no background signal was observed when the expression plasmids for L, N, and P were omitted (Fig. 2C, lane 17). Thus, the helpervirus-free Pol I-based system demonstrates a very good signal-to-noise ratio.

Role of the support plasmid L : N : P ratios on minigenome rescue efficiency

To further characterize the established Pol I-driven NiV minigenome rescue system and to find conditions for the most efficient rescue rates, we tested select ratios of the three viral protein expression plasmids NiV L, N, and P in combination with 1.0 μg of the antigenomic (cRNA-oriented) minigenome pPol I NiV-CAT (+). Previously published amounts of L, N, and P expression plasmids used with the T7-based approach of 0.2 μg, 0.6 μg, and 0.4 μg (molar ratio L : N : P of 0.3 : 1.7 : 1.0, respectively) (Halpin et al., 2004), have been sub-optimal in our hands (Fig. 3A: lanes 9, 22, 28, 46). To determine the plasmid ratios for the most efficient minigenome rescue, and to study the requirement of *trans*-acting factors for optimal NiV minigenome transcription and replication, amounts of two support plasmids were kept constant while the third was varied. In total, we tested 40 combinations of expression plasmid ratios (Fig. 3A). Negative controls (omitting one of the support plasmids) were always included in a group of six different conditions of all NiV support plasmids (first lane in each experiment). Relative CAT activities were evaluated using the TINA 2.09 program (Raytest) (Fig. 3B). The molecular ratios of the transfected L : N : P plasmids were calculated considering the sizes of 12100 nt, 6981 nt and 7514 nt of the L, N, and P expression plasmids, respectively. We conclude that the Pol I-driven minigenome system is sensitive to minor changes in the ratios of the support plasmids, as even slight variations resulted in major changes in CAT reporter expression levels. This suggests that NiV minigenome transcription and replication is highly dependent on optimal amounts and ratios of transfected NiV L, N, and P expression plasmids. Interestingly, more than one ratio of the three support plasmids that resulted in notably stronger minigenome expression were identified. Subsequently, we tested conditions resulting in peak levels from the titration study in parallel for better comparison (Fig. 3C: lanes 2 to 14). Additionally, these results were directly compared to the T7-driven minigenome rescue system (Fig. 3C, lane 1). Higher amounts of NiV P (0.2 versus 0.4 μg) seem to have an inhibiting effect on minigenome expression (Fig. 3A: lanes 38 to 42 versus 44 to 48), while the amount of NiV N seems not to be critical in the tested range (0.3 and 0.6 μg; e.g., Fig. 3A: lanes 14 to 18 versus 20 to 24). The same also applies to the amount of NiV L, as no difference in reporter gene

activity is detectable within the tested range (0.2 and 0.4 μg; Fig. 3A: lanes 26 to 30 versus 32 to 36). Overall, the transfection of more than 0.6 μg of NiV P or less than 0.1 μg of NiV L and 0.2 μg NiV N did not result in any detectable minigenome expression, suggesting that excess NiV P interferes with efficient NiV minigenome transcription/replication, whereas a minimal amount of NiV L and N is required for minigenome expression.

Establishment of a high-throughput screening assay for potential antiviral compounds

After development, characterization, and optimization of T7- and Pol I-driven NiV minigenome rescue systems, we downscaled both for use in a screening assay for potential antiviral compounds. Since classical thin layer chromatography-based CAT assays are not suitable for high-throughput assays, a CAT ELISA approach was used instead. For this, minigenome rescue was characterized and optimized using 24-, 48- and 96-well plates to determine sensitivity and downscaling potential of the established reporter systems. Transfecting minigenome-expressing plasmids (0.1 μg of pPol I NiV-CAT (+) or pT7 NiV-CAT (-)) and NiV L (0.1 μg), N (0.15 μg), and P (0.05 μg) protein-expressing plasmids (either under T7- or CMV-control) along with 0.25 μg pCAGGS/T7 in 293T/17 cells resulted in strong and reproducible signals after an incubation time of 48 h. This was confirmed by both enzymatic CAT reactions (data not shown) and CAT ELISAs (Fig. 4A). These results suggest that the NiV minigenome rescue systems have appropriate signal-to-noise ratios as well as suitable sensitivity to be used in a 96-well-based high-throughput screening assay for antiviral drugs.

Next, we validated the new system to prove our concept of using the NiV minigenome rescue platform as a high-throughput screen. Transfected 293T/17 cells in a 96-well format were treated with potential antiviral compounds (at a concentration of 20 μM) for 48 h, and assayed for CAT activity. The analyzed compounds are part of the 1,990 compounds NCI Diversity Set Library (kindly provided by M. Javad Aman, USAMRIID, Fort Detrick, MD). We limited our initial analysis to 80 samples to assess the suitability, efficacy, and practicability of our assay. As controls, non-transfected (Fig. 4B, A1-H1 representing column number 1 of a 96-well plate) and transfected cells (Fig. 4B, A12-H12 representing column number 12 of a 96-well plate) were incubated with 1% DMSO, the solvent for the compounds. Compounds showing inhibitory effects resulting in a 50% decreased CAT activity (≤ OD 1.0) were considered to exhibit anti-NiV activity. Two compounds that decreased reporter gene activity could be identified (Fig. 4B: wells A7 and F9). Cytotoxic effects of the compounds inhibiting NiV minigenome expression were excluded as shown by a viability test based on Trypan blue staining (data not shown). Nevertheless, a lower level of reporter gene expression could also be explained not only by a NiV-specific mechanism but also by the possibility that potential antiviral compounds alter the translation of the reporter gene or the co-transfected support proteins. Subsequent experiments using live virus under BSL-4 conditions will determine the specificity of the identified potential antiviral compounds.

To confirm our initial hits and to exclude false positive hits that simply interfere with the Pol I-based reverse genetics technology, the candidate compounds A7 and F9 were subsequently tested with our helpervirus-free T7-driven NiV minigenome rescue platform (Fig. 4C). Interestingly, compound F9 showed a slightly stronger influence decrease of the CAT activity with the T7-driven minigenome system. As a positive control we used compounds A2 and D3, which had shown no effect in the Pol I-driven minigenome screen. Both, the T7 polymerase as well as the Pol I-driven minigenome screening platform, yielded similar results, therefore confirming the interfering activity of Nipah-viral transcription and replication of some of the tested compounds.

In summary, the results presented in this study demonstrate the suitability, efficacy, and practicability of the two plasmid-based NiV minigenome rescue systems in a 96-well plate

format. This format can be used as high-throughput screening platforms for antiviral compound libraries to identify molecules that interfere with Nipah viral transcription and replication processes.

Discussion

Previously reported minigenome rescue systems for members of the family *Paramyxoviridae* have been based exclusively on a bacteriophage T7 polymerase-driven reverse genetics technology which requires the introduction of the exogenous T7 polymerase (Conzelmann and Schnell, 1994; Grosfeld, Hill, and Collins, 1995; Kato et al., 1996; Radecke et al., 1995; Stillman, Rose, and Whitt, 1995). While this can be achieved in several ways, including recombinant vaccinia virus infection and transient expression from plasmid DNA (Fuerst et al., 1986; Neumann et al., 2002; Theriault et al., 2004), this step has the potential to limit the system's efficiency due to technical difficulties in achieving T7 expression in all cells. In contrast, Pol I is a eukaryotic host cell polymerase which is localized in the nucleoli, transcribing mainly rRNA genes (Sollner-Webb and Tower, 1986). Using this enzyme to drive a reverse genetics system provides a substantial advantage because it alleviates the need to supply the polymerase in *trans*. The localization of Pol I in the nucleus is one potential limitation of such a system, especially for its application to viruses which replicate exclusively in the cytoplasm. However, it was previously employed successfully for the development of minigenomes for several members of the *Bunyaviridae* (Flick et al., 2003a; Flick et al., 2003b; Flick and Pettersson, 2001), *Arenaviridae* (Lee et al., 2000), as well as for *Filoviridae* (Groseth et al., 2005) that all replicate in the cytoplasm. Together with the data presented herein, this indicates that Pol I-driven transcription is a broadly applicable system for minigenome rescue, even for viruses not replicating in the nucleus.

In the present study we described the establishment of an RNA polymerase I-based minigenome rescue system for the recently emerged *Paramyxovirus* NiV and the use of this system as a high-throughput screening platform for potential antiviral drugs interfering with viral transcription and replication processes. To the best of our knowledge, this is the first report demonstrating the successful use of a Pol I reverse genetics technology for a member of the *Paramyxoviridae*, and only the second for a nonsegmented negative-strand RNA virus after Reston ebolavirus (Groseth et al., 2005). However, a recent study demonstrated the successful rescue of Borna disease and measles virus, both members of the *Mononegavirales*, by using RNA polymerase I- and II-mediated expression of viral cRNA (Martin, Staeheli, and Schneider, 2006).

Here we demonstrated transcription and replication of Pol I-generated NiV minigenomes by the viral proteins L, N, and P supplied by transfection of the corresponding expression plasmids. Both genomic- and antigenomic-oriented minigenomes were rescued as demonstrated by strong CAT activity in transfected cells (Figs. 2A and B). The antigenomic minigenome produced comparable reporter gene activity to the minigenome in genomic orientation (Fig. 2B). This is consistent with observations with other *Mononegavirales* such as Sendai virus (Calain and Roux, 1993) or Ebola and Marburg virus, where minigenome rescue could be achieved using cRNA-oriented minigenomes (Mühlberger et al., 1998; Mühlberger et al., 1999). In fact, the first successful rescue of infectious rabies virus entirely from cDNA was made possible only by the use of a complete antigenome instead of genomic-oriented RNA (Schnell, Mebatsion, and Conzelmann, 1994). Since then, most full-length rescue systems for *Mononegavirale*s were based on antigenomic cDNA clones (Conzelmann, 2004; Enterlein et al., 2006; Kawaoka, 2004; Neumann, Whitt, and Kawaoka, 2002; Walpita and Flick, 2005). The here described study also demonstrates that the antigenomic minigenomes produced virtually no background signal even at high input levels (Fig. 2B) and long incubation times (Fig. 2C).

In order to evaluate the potential of the Pol I-driven minigenome rescue system as an alternative to the more common T7-driven platform, both systems were compared directly. When equal amounts of both minigenome plasmids (vRNA-oriented) were transfected along with the required support plasmids, the Pol I-derived minigenomes produced higher reporter gene expression levels compared to the T7-driven construct (Fig. 2A, lane 6 and Fig. 1B, lane 2). In the absence of protein expression plasmids, the level of CAT expression from the minigenome plasmid alone was less with the Pol I-driven system than that of the T7-driven minigenome, demonstrating a better signal-to-noise ratio for the Pol I system. Good signal-to-noise ratio is desirable for subsequent applications (e.g., determining time points for maximum reporter gene activity or different ratios of viral protein support plasmids). Based on these data, Pol I appears to be an attractive alternative to T7 for the development of minigenome rescue systems. Additionally, this technology eliminates the need for two inefficient steps associated with a T7 system, namely the introduction of an exogenous polymerase and ribozyme cleavage to generate precise transcript ends.

Titration experiments with the support plasmids NiV L, N, and P showed that replication and expression of the NiV minigenome is affected most severely by increasing amounts of NiV P (Fig. 3A: lanes 38 to 42 and 44 to 48). For other members of the *Mononegavirales*, including Marburg virus (Mühlberger et al., 1998), Sendai virus (Kato et al., 1996), and Borna disease virus (Perez et al., 2003;Schneider et al., 2003), it was shown that the ratio between P and N was critical for reporter gene expression. In contrast, the amount of NiV N and NiV L input DNA seems to be of less importance for minigenome rescue (Fig. 3A). Comparable results have been obtained with Marburg virus (Mühlberger et al., 1998) and Sendai virus (Kato et al., 1996).

In addition to the successful development of a Pol I-driven system, we also established a helpervirus-free, completely plasmid-driven T7 RNA polymerase-based approach by using (*i*) stable expressing BHK-T7 cells or (*ii*) transfected T7 expression plasmids.

As an application, we used both approaches to establish a high efficient and reliable screening system for potential antiviral compounds that interfere with the Nipah-viral transcription and replication processes. Due to the absence of vaccines or chemotherapeutic agents for the treatment of NiV and HeV infections, and the recently described potential for person-to-person transmission during a NiV outbreak in India/Bangladesh (Chadha et al., 2006), it is of high priority to develop assays that allow high-throughput screening for potential antivirals. With the described NiV reverse genetics systems, we have established tools which can be used for high-throughput screening of libraries containing thousands of potential inhibitors. The advantages of such a system are (*i*) lack of infectious material thus no restriction to high containment laboratories, (*ii*) the possibility of identifying antiviral compounds with a broad spectrum of antiviral activity, (*iii*) viral and/or host cell pathways necessary for viral replication can be targeted, and (*iv*) compound-related toxicity is readily detectable.

The concept of the present technology is based on the transfection of cells with the NiV minigenome system which results in the expression of CAT reporter gene directly reflecting the extent of viral replication. Monitoring the suppression of the reporter gene activity would allow the identification of antiviral compounds targeting replication and/or transcription of NiV. One important advantage of using two minigenome rescue systems (Pol I and T7) for screening large libraries is to reduce false hits that are related to the respective expression strategy and are not virus-specific. Additionally, the two alternative minigenome systems are also valuable tools for basic research, e.g., identifying *cis*-acting genomic elements, studying virus encapsidation, transcription, replication, and genome packaging, while eliminating biosafety constraints required for work with infectious material. Although an infectious clone

system has been described for NiV recently (Yoneda et al., 2006) the minigenome systems are invaluable tools to allow research in BSL-2 laboratories.

Recently, two studies described the formation of NiV virus-like particles (VLPs) when expressing NiV matrix, fusion and attachment proteins (Ciancanelli and Basler, 2006; Patch et al., 2007). With the fully characterized minigenome rescue system under Pol I and T7 control presented in this study, the next step would be the establishment of a VLP system containing the minigenome as a reporter to screen for antivirals that e.g., interfere with virus-receptor interactions.

In this study we only demonstrated the scalability of the assay to high throughput format as proof-of-concept. However, development and validation of a functional and robust drug screening assay will require further optimizations that are beyond the scope of this study and will be pursued in the future. Further investigations of the potential antiviral drugs found in this study, including dose-dependent titrations and confirmative live virus studies, will also be performed.

Material and Methods

Cell lines

293T/17 (a derivative of the human embryonic kidney cell line 293T) and HeLa (human epithelial) cells (obtained from the American Type Cell Culture) were cultivated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen), supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 IU penicillin/ml, and 100 µg streptomycin/ml (Cellgro). T7 polymerase stably-expressing baby hamster kidney cells (BHK-T7 724-5cl) were kindly provided by I. V. Frolov (University of Texas Medical Branch, Department of Microbiology & Immunology, Galveston, TX). These cells were cultivated in Alpha Modified Eagle Medium (α-MEM; Invitrogen), supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Invitrogen), 10 µg puromycin/ml (Sigma Aldrich), 100 IU penicillin/ml, and 100 µg streptomycin/ml (Cellgro).

Plasmid construction

Nipah virus minigenome plasmids

Plasmids designed for transcription of NiV genomic [vRNA, (-)] or antigenomic [cRNA, (+)] RNA by the cellular Pol I contain the human Pol I promoter and the mouse Pol I terminator (Flick et al., 2003b; Neumann et al., 1999; Zobel, Neumann, and Hobom, 1993). The NiV minigenome, a CAT reporter gene flanked by the two non-coding regions (nucleotides 18246-18147 of the 5′ trailer sequence and nucleotides 1-115 of the 3′ leader sequence), was amplified by PCR using pNiV-CAT (Halpin et al., 2004) as a template (kindly provided by P. Rota, Center for Disease Control and Prevention). Oligonucleotides LD165/166 and LD167/168 were used for amplification of the minigenomes in either genomic or antigenomic orientation, respectively (Table 1). After cleavage with BbsI, the PCR fragments were inserted between the Pol I promoter and terminator elements of pLD 9, a vector similar to the previously published pRF42 (Flick and Pettersson, 2001) containing the human Pol I promoter (unpublished data, L. Deflubé and R. Flick). The resulting constructs were designated pPol I NiV-CAT (-) and pPol I NiV-CAT (+).

Nipah virus protein expression plasmids under CMV control

The NiV N and P ORFs were amplified by PCR from the plasmids pNiV-N and pNiV-P (kindly provided by P. Rota, Center of Disease Control and Prevention) (Halpin et al., 2004) using primers LD181/182 and LD179/180, respectively. Expression of the non-structural proteins C, V, and W from the P gene was suppressed by silencing the C ORF through mutation of the

two consecutive start codons to ACG (Halpin et al., 2004). Both the N and P ORFs were cloned into expression vectors under the Cytomegalovirus (CMV) immediate early promoter and enhancer element (pcDNA 3.1; Invitrogen) to yield pCMV NiV-N and pCMV NiV-P, respectively. The integrity of all constructs was verified by automated sequencing over the full PCR amplification product and adjacent flanking sequences (Macrogen Inc.).

Transfection

Sub-confluent cells (80 to 90%) seeded in tissue culture 24-well plates were transfected with NiV minigenome plasmids together with NiV L, N, and P expression plasmids. Transfection was carried out using Lipofectamine 2000 (Invitrogen Carlsbad, CA) following the manufacturer's protocol. Briefly, DNA was mixed with 20 μl Opti-MEM (Invitrogen, Carlsbad, CA) and 2 μl of transfection reagent, and incubated for 20 min at room temperature. Subsequently, cells were incubated with the DNA/Lipofectamine mixture and 100 μl of Opti-MEM for 3 h, whereupon 1 ml of fresh medium with 2.8% FBS was added. CAT activity was evaluated at 48 h post-transfection if not stated otherwise. Negative controls omitting one of the expression plasmids were compensated with pTM1. The plasmid pHL2823 (R. Flick and G. Hobom, unpublished data), which contains the enhanced GFP ORF under control of a CMV promoter was transfected as a transfection efficiency control in all performed experiments.

CAT assay

Cells from 24-well plates were harvested in 1 ml of PBS, pelleted in a F45-24-11 rotor (5 min at 2800 rpm, Eppendorf), resuspended in 55 μl of 250 mM tris·hydrochloride (pH 7.4) and lysed by three freeze-thaw cycles. Cell lysates were incubated at 56 °C for 10 min. Subsequently, cells were spun for 10 min at 13,200 rpm. An enzymatic CAT reaction (FAST CAT Kit; Invitrogen) was performed by mixing the lysate with 10 μl of 9 mM Acetyl Coenzyme A (Sigma Aldrich), 10 μl Component A (Fast CAT Kit; Invitrogen) and 0.25 M tris-hydrochloride (pH 7.4) to a final volume of 75 μl. After an incubation time of 4 to 16 h at 37 °C samples were prepared for thin-layer chromatography according to the FAST CAT Kit protocol. The reaction products were visualized by UV-illumination and documented by photography with a Gel Doc 2000 system (BioRad). Relative CAT activity was evaluated using the TINA 2.09 program (Raytest).

Antiviral compounds and assay for specificity screening

The 80 tested antiviral compounds, kindly provided by M. Javad Aman (United States Army Medical Research Institute for Infectious Diseases), are part of the NCI Diversity set library. This library represents a collection of 1,990 compounds accumulated out of 140,000 compounds available on plates. These compounds were dissolved in DMSO and assayed for their antiviral effect by using both, the pT7 NiV-CAT (-) and pPol I NiV-CAT (+) minigenome plasmids, in 96-well plates. Sub-confluent 293T/17 cells were transfected with the NiV minigenome plasmid and the NiV L, N, and P expression plasmids as described earlier. Three hours post-transfection the antiviral compounds were added in a 1:10 dilution (20 μM final concentration) and incubated for 48 h. As control reactions, untransfected (negative control) and transfected (positive control) cells were treated only with the solvent DMSO (1%). Minigenome expression levels were evaluated by using a CAT enzyme-linked immunosorbent assay (ELISA) kit (Roche).

CAT ELISA

Quantification of CAT gene expression from 96-well plate experiments was performed using a CAT ELISA kit (Roche) following the manufactures's protocol. Briefly, two days post-transfection, cells were lysed in 100 μl of CAT lysis buffer for 1 h at room temperature, and subsequently cell lysates were transferred into individual wells of a 96-well plate coated with

an anti-CAT specific monoclonal antibody (Mab) and incubated for 1 h at 37 °C. The wells were washed extensively with washing buffer, and a second CAT-specific Mab labeled with digoxigenin (DIG) was added to each well. After 1 h incubation at 37 °C wells were washed again and incubated with a peroxidase-coupled DIG-specific Fab fragment for 1 h at 37 °C. In a last step, peroxidase-substrate (without substrate enhancer) was added, resulting in a peroxidase-mediated color reaction. The colored product, which is directly correlated to the level of CAT activity, was quantified spectrophotometrically at a wavelength of 405 nm after an incubation time of around 20 min at room temperature using a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA). We defined compounds as potential antiviral drugs (at the used test concentration of 20 μM) as such whose effects resulted in a 50% decrease in reporter gene activity at 405 nm compared to the average value of all tested compounds (corresponds to an OD at 450 nm of 1.0 compared to the average value of 2.0 ± 0.37).

Viability test

293T/17 cells were plated in a 96-well plate as described above, and antiviral compounds are added in a final volume of 100 μl medium and harvested 48 h post-exposure. Trypan blue staining was performed to distinguish between viable from non-viable cells. For this, 25 μl of cells were diluted with 5 μl of the dye and counted in a hemacytometer chamber. The percentage of viable cells was calculated by using the following formula:

$$\%\text{Viability} = (\text{\# viable cells counted/total \# cells counted}) \times 100$$

Acknowledgments

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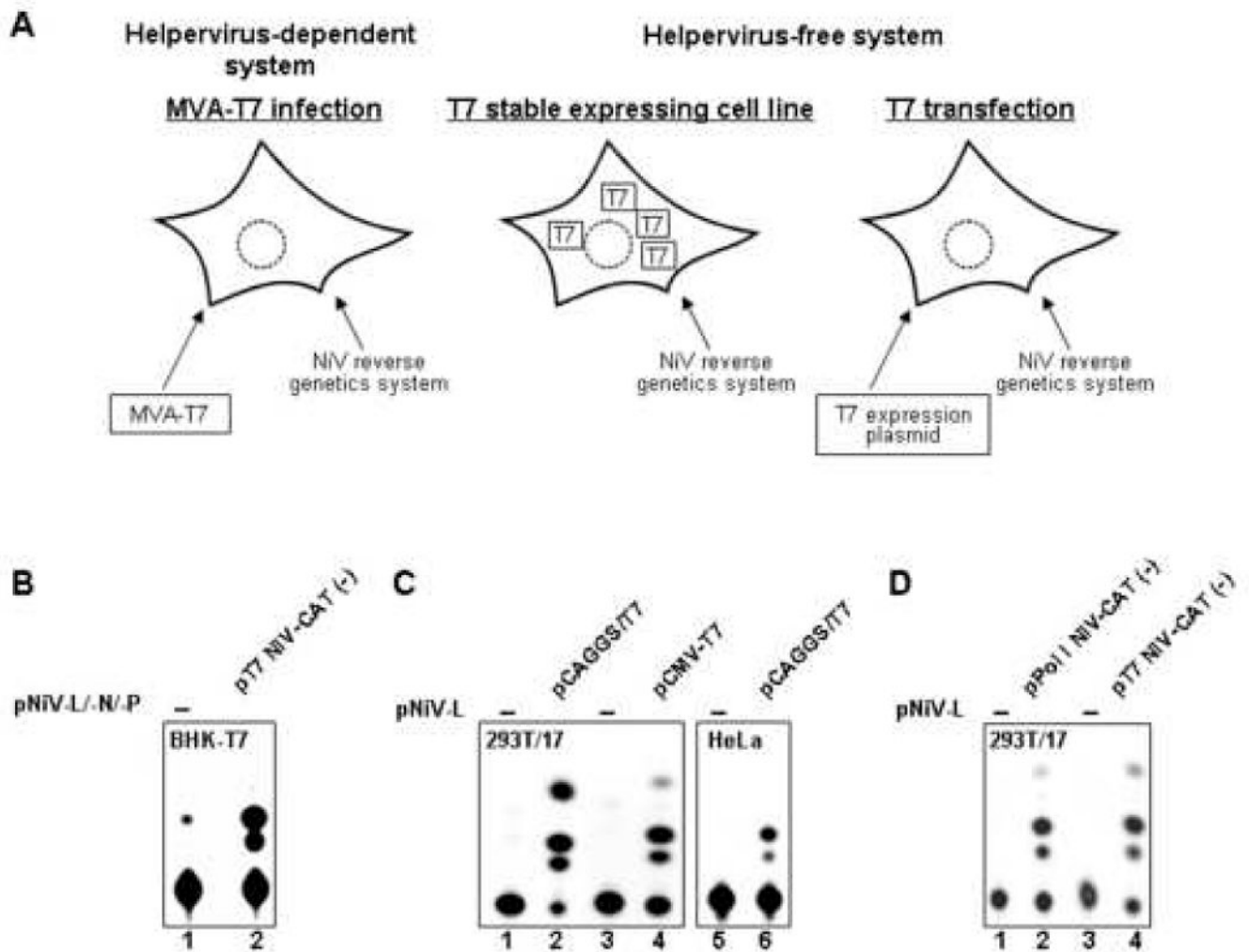
Fig. 1. Establishment of a helpervirus-free T7 RNA polymerase- or Pol I-driven NiV minigenome rescue systems
(A) Schematic representation of different strategies to deliver the T7 RNA polymerase for the T7 RNA polymerase-driven minigenome rescue system. (B) Helpervirus-free T7 RNA polymerase-driven NiV minigenome rescue. BHK-T7 cells in 24-well plates were transfected with 0.2 µg of minigenome and constant amounts of T7-driven support plasmids (NiV L, 0.2 µg; NiV N, 0.6 µg; NiV P, 0.4 µg). Lane 1 represents the negative control without co-transfected NiV L, N, and P. (C) Comparison of two different sources for T7 RNA polymerase expression in a helpervirus-free and completely plasmid-driven T7-based NiV minigenome rescue system. 293T/17 cells were co-transfected with pT7 NiV-CAT (-) (0.2 µg) and all NiV expression plasmids (NiV L, 0.2 µg; NiV N, 0.6 µg; NiV P, 0.2 µg) under T7-control along with 2 µg of pCAGGS/T7 (lanes 1 and 2) and pCMV-T7 (lanes 3 and 4), respectively. Lanes 5 and 6 show transfection of the above mentioned NiV minigenome rescue system plasmids along with pCAGGS/T7 in HeLa cells. In negative controls NiV L was omitted. (D) Comparison of T7- and Pol I-driven NiV minigenome rescue systems. 293T/17 cells were either transfected with 0.5 µg pPol I NiV-CAT (-) or 1.0 µg pT7 NiV-CAT (-) and co-transfected with support plasmids under either T7- or CMV-control (NiV L, 0.2 µg; NiV N, 0.6 µg; NiV P, 0.2 µg). In all experiments 1 µg of pCAGGS/T7 was included as the source for the T7 RNA polymerase. In negative controls NiV L was omitted.
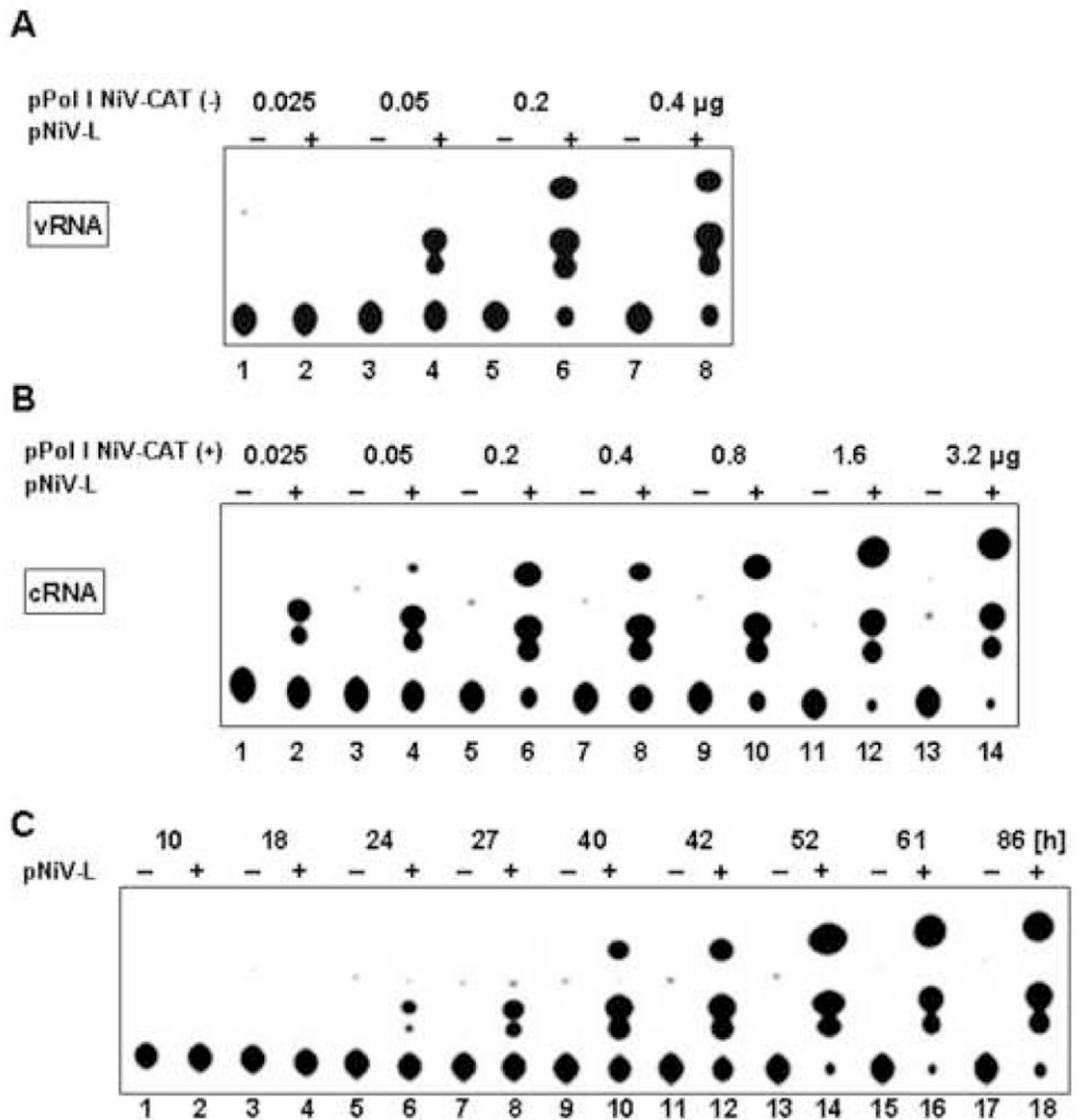
Fig. 2. Characterization of RNA polymerase I-driven NiV minigenome rescue system
293T/17 cells in 24-well plates were transfected with increasing amounts of pPol I NiV-CAT
minigenome plasmids [A: vRNA-oriented (genomic); B: cRNA-oriented (antigenomic)],
constant amounts of support plasmids (NiV L, 0.2 μg; NiV N, 0.6 μg; NiV P, 0.2 μg), and
pCAGGS/T7 (1 μg). In negative controls (odd lanes) NiV L was omitted. (C) Time course
experiment to determine most efficient NiV minigenome rescue. 293T/17 cells were
transfected with 1 μg pPol I NiV-CAT (+) minigenome plasmids and the NiV support plasmids
as described above. Cells were harvested at different time points post-transfection (10 to 86 h)
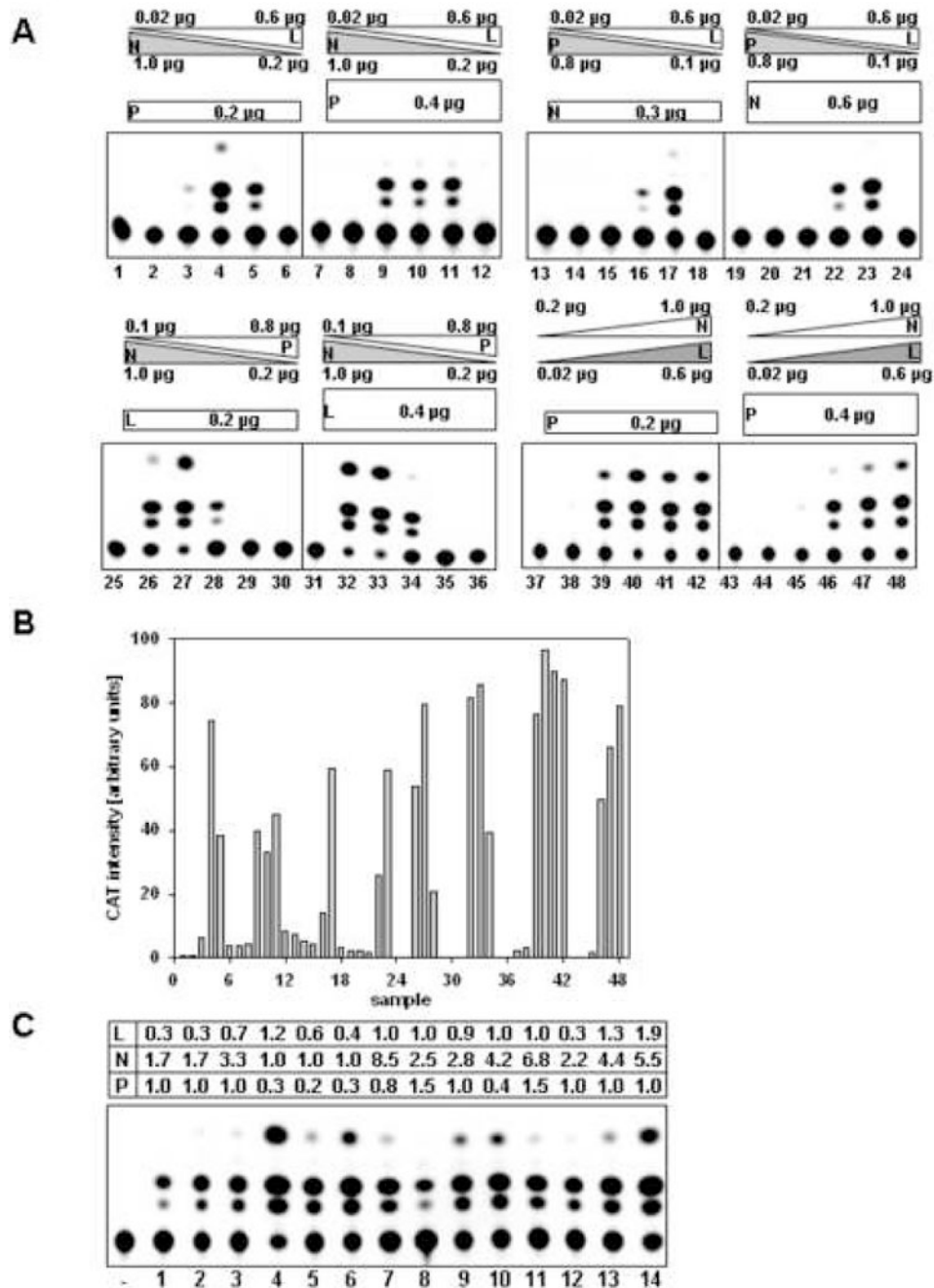and analyzed for reporter gene activity. In negative controls NiV L was omitted (odd lanes).

Fig. 3. Determination of *trans*-acting factors required for NiV minigenome transcription and replication

(A) 293T/17 cells were transfected with various amounts of support plasmids expressing NiV L, N, and P, along with 1 μg of Pol I-driven cRNA-oriented NiV-CAT minigenome and 1 μg of pCAGGS/T7. For one NiV support expression plasmid the amount of transfected DNA was kept constant, while the amount for the other two was varied. Two different amounts of DNA were chosen for the expression plasmid that was kept constant. In the negative controls (always the first lane in a set of six different conditions) one of the expression plasmids (NiV L, N, or P) was omitted. (B) Diagram of relative percentage of CAT activities measured in A. Relative CAT activities of each single condition was evaluated using the TINA 2.09 program (Raytest).

(C) Direct comparison of NiV minigenome expression levels using optimal ratios and amounts for NiV L, N, and P expression plasmids. Lane 1 represents the completely T7-based and lanes 2 to 14 the Pol I-based NiV minigenome rescue system. The table above the CAT assays represents the molar ratios between NiV L : N : P.
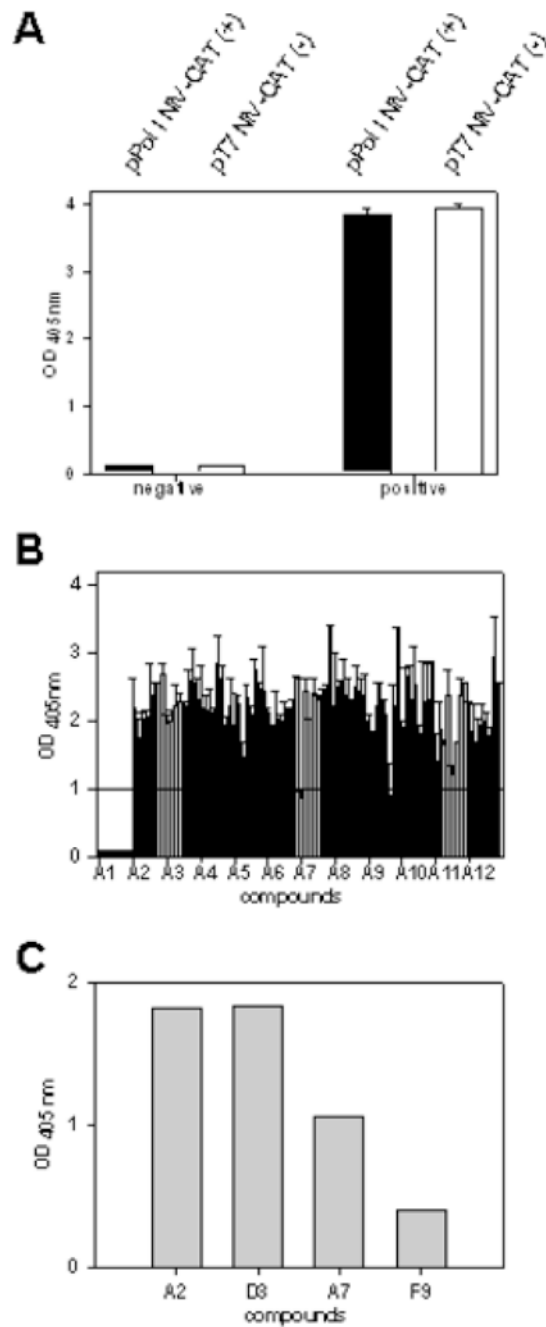
Fig. 4. Establishment of high throughput screening assays to identify antivirals against NiV
(A) Minimization of NiV minigenome rescue systems. 293T/17 cells in 96-well plates were
co-transfected with 0.5 μg pPol I NiV-CAT (+) or pT7 NiV-CAT (-) minigenome plasmids,
pCAGGS/T7 (0.25 μg), and NiV expression plasmids under either T7- or CMV-control (NiV
L, 0.1 μg; NiV N, 0.15 μg; NiV P, 0.05 μg). Reporter gene activity was measured using a CAT
ELISA Kit (Roche). Black bars indicate the pPol I NiV-CAT (+) and white bars the pT7 NiV-
CAT (-) minigenome. Negative controls omitting NiV L are labeled with as negative. Error
bars for the positive controls were calculated out of 8 individual experiments. (B) Diagram of
relative CAT activities of pPol I NiV-CAT (+) minigenome plasmid after treatment with
antiviral compounds (20 μM final concentration). CAT activities were measured at 405 nm

using the CAT ELISA Kit (Roche). Columns of a 96-well plate are displayed beside each other on the x-axis of the diagram. Negative (A1-H1, untransfected cells) and positive (A12-H12, transfected cells) control reactions were performed by treating cells with DMSO. The error bars were calculated out of 3 individual experiments. (C) Diagram of relative CAT activities of pT7 NiV-CAT (-) minigenome plasmid after treatment with potential antiviral compounds measured at 405 nm using the CAT ELISA Kit (Roche).

Table 1

Oligonucleotides used for plasmid construction

primer	sequence (5′→3′)	cloning product
LD165	AAT**GAAGAC**GGTATT<u>ACCGAACAAGGGTAAAGAAGAATCG</u>	pPol I NiV-CAT (-) [vRNA]
LD166	AAT**GAAGAC**TTGGGG<u>ACCAAACAAGGGAGAATATGGATACG</u>	
LD167	AAT**GAAGAC**TTGGGG<u>ACCGAACAAGGGTAAAGAAGAATCG</u>	pPol I NiV-CAT (+) [cRNA]
LD168	AAT**GAAGAC**GGTATT<u>ACCAAACAAGGGAGAATATGGATACG</u>	
LD179	AAT**CTCGAG**CCATG<u>GATAAATTGGAACTAGTCAATG</u>	pCMV NiV-P
LD180	AAT**GGATCC**TCA<u>AATATTACCGTCAATGATGTC</u>	
LD181	AAT**GAAGAC**GGTCGACCATG<u>AGTGATATCTTTGAAGAGGC</u>	pCMV NiV-N
LD182	AAT**GAAGAC**GGGATCTCA<u>CACATCAGCTCTGACGAAATC</u>	

Boldface: endonuclease restriction recognition sequences; underlining: NiV NCR sequences.