Inhibition of Marburg virus protein expression and viral release by RNA interference

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High mortality rates and lack of an available vaccine against Marburg haemorrhagic fever (MHF) highlight the need for a defensive therapy against MHF and greater knowledge of the causative agent, the Marburg virus (MARV). Here, RNA interference (RNAi) is employed to destroy MARV transcripts, disrupting replication and allowing analysis of various roles of MARV proteins.

Small interfering RNAs (siRNAs) homologous to three MARV transcripts (NP, VP35 and VP30) were co-transfected into cells with plasmids encoding the corresponding nucleocapsid proteins. The resulting decrease in MARV nucleocapsid-protein levels was shown to be specific, as siRNA that was not homologous to the MARV genome did not decrease the levels of viral nucleocapsid proteins. Additionally, transcript levels of double-stranded RNA (dsRNA)-sensor proteins, the dsRNA-activated protein kinase and 2′,5′-oligoadenylate synthetase 1 remained unchanged, suggesting that the decrease in viral proteins was not a result of activation of the antiviral properties of the interferon system. Subsequently, siRNAs were shown to reduce intracellular viral proteins in MARV-infected cells and viral material released into the medium.

Targeted reduction of VP30 downregulated the intracellular levels of all other viral proteins, suggesting that VP30 plays an essential role for transcription/replication. The efficient reduction of MARV replication also suggests that RNAi may provide an agent against MHF.

INTRODUCTION

RNA interference (RNAi) is a post-transcriptional gene-silencing process in which double-stranded RNA (dsRNA) initiates specific cleavage of cytoplasmic mRNA. Initially, dsRNA is recognized and processed by an RNase III enzyme, commonly referred to as DICER (Hutvágner et al., 2001). The resulting processed dsRNA consists of 21–23 nt long dsRNA fragments with 2–3 nt overhangs at the 3′ end of each RNA strand (Zamore et al., 2000; Bernstein et al., 2001). DICER-processed dsRNA is recognized by the dsRNA-induced silencing complex (RISC) and used as a template to guide splicing of mRNA that is homologous in sequence to the RISC-bound dsRNA fragment, resulting in greatly reduced protein production (Hammond et al., 2000).

Cellular recognition of dsRNA is associated with viral infection and engagement of the interferon-response system. Produced during virus replication, dsRNA can be recognized by the cell, resulting in the release of interferon into the extracellular milieu, which can be received by neighbouring cells as a signal to initiate virus-defence measures. Activated by interferon receptor–signalling pathways, the RNA-dependent protein kinase (PKR) and 2′,5′-oligoadenylate synthetase 1 (OAS-1) can also be activated by binding dsRNA, resulting in inhibition of translation and mRNA degradation, respectively, and an increase in transcription of both molecules [reviewed by Stark et al. (1998)]. However, the small size of dsRNA (21–23 nt) that is recognized by RISC and employed in RNAi is believed to avoid PKR and OAS-1 detection, which is reported to require a minimal sequence size of 30 nt (Manche et al., 1992; Elbashir et al., 2001a), thus avoiding engagement of a general, non-specific shutdown of protein production and mRNA degradation.

Synthetically produced small interfering dsRNA molecules (siRNAs) have been shown to induce the RNAi effect in vitro and greatly decrease specifically targeted transcripts (Elbashir et al., 2001b). Such efforts have been expanded to include targeting of viral transcripts present in the cytoplasm for degradation by the RISC complex. A short list of the viral pathogens that have been shown to be susceptible to an in vitro RNAi-directed attack includes human immunodeficiency virus, hepatitis B virus, influenza
A virus and human papillomavirus [reviewed by Saksela (2003)].

Classified in the order Mononegavirales, Marburg virus (MARV) is capable of causing haemorrhagic fever, with mortality rates ranging from 30 to 90 % (Feldmann & Kiley, 1999). The MARV virion is composed of seven structural proteins. Four of them, NP, VP35, VP30 and L, together with the viral RNA genome, form a helical nucleocapsid (Becker et al., 1998; Mühlberger et al., 1998). This nucleocapsid is encased in a lipid bilayer derived from the host-cell plasma membrane, which also displays the virus-produced transmembrane glycoprotein GP (Will et al., 1993). A matrix composed of the proteins VP40 and VP24 connects the nucleocapsid with the lipid envelope (Becker et al., 1998). The genome of MARV is represented by a negative-sense, single-stranded RNA molecule that contains the seven genes arranged in a linear order: 3’-NP-VP35-VP40-GP-VP30-VP24-L-5’ (Feldmann & Kiley, 1999).

During the replication cycle of MARV, the encapsidated genome is replicated into full-length antigenomes that in turn serve as templates for the synthesis of genomes that are incorporated into progeny virions. The viral genome is also transcribed into subgenomic RNA molecules that are not encapsidated, but can be translated by the cellular translation machinery into MARV-specific proteins (Mühlberger et al., 1998).

Here, we show that, by using an RNAi-based approach, transiently expressed MARV nucleocapsid proteins NP, VP35 and VP30 were efficiently silenced. Moreover, silencing was also successful in MARV-infected cells. Decreasing the cellular levels of NP, VP35 and VP30 in MARV-infected cells invariably resulted in a decreased release of progeny virions in the supernatant. Surprisingly, downregulation of VP30, whose function for MARV replication and transcription is so far unknown, had a definite impact on the intracellular levels of all other MARV proteins tested.

**METHODS**

Molecular cloning and siRNA. Molecular cloning of MARV sequences followed standard procedures. Full-length open reading frames of VP35, VP30 or NP (GenBank/EMBL accession no. Z12132) were cloned from pT-NP, pT-VP35 and pT-VP30 plasmids (Mühlberger et al., 1998) into expression vector pCAGGS (Niwa et al., 1991) by virtue of engineered restriction-enzyme sites: EcoRI and Stul for VP35, Stul for VP30 and EcoRI for NP. Generated plasmids pC-VP30, pC-VP30 and pC-NP were verified by automated sequencing. siRNA oligonucleotides with 3’-dT extensions (Table 1) were synthesized against the three MARV sequences mentioned above, human lamin A/C (accession no. M13451) and CD44 (accession no. U40373) by Qiagen. As a negative control, we used an siRNA (siX, Qiagen; Table 1).

HeLa CCL-2 and Vero cell assays. The indicated amount of plasmids encoding MARV nucleocapsid proteins with or without siRNA were incubated with Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). HeLa CCL-2 cells and Vero cells were maintained in full medium: RPMI 1640, 25 mM HEPES, 2 mM l-glutamine (Invitrogen) supplemented with 10 % fetal calf serum (FCS; Biochrom KG), 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (Gibco) in six-well plates (Corning). After removing medium from ~70 % confluent monolayers, 500 μl Opti-MEM I (Invitrogen) was added to cells. Aliquots (100 μl) of Lipofectamine 2000 mixtures containing plasmids and/or siRNA were added to cells and incubated at 37 °C, 5 % CO₂. After 4 h, mixtures were removed from cells and full medium was added. Fresh medium was reapplied after 24 h and cells were harvested at 48 h post-transfection unless indicated.

To harvest cell lysates, medium was removed and cells were washed once with sterile Dulbecco’s PBS (Gibco). Cells were trypsinized and washed with full medium, followed by centrifugation and washing with cold PBS. After removal of PBS, 100 μl cell-lysis buffer [50 mM Tris/HCl (pH 7−5), 150 mM NaCl, 1-0 % NP 40, 1 μg aprotinin ml⁻¹, 250 μg Pefabloc ml⁻¹ (Roche), 0-7 μg pepstatin ml⁻¹ and 0-5 μg leupeptin ml⁻¹] was added to cell pellets. Pellets were resuspended and incubated on ice for 45 min. The resulting lysates were stored at −20 °C until ready for use. After thawing on ice and centrifugation, lysate protein concentrations were quantified by measuring A₂₅₀ with Bradford solution (Sigma). Then, SDS-PAGE loading buffer [200 mM Tris/HCl (pH 6−8), 8-0 % SDS, 0-2 % bromophenol blue, 40 % glycerol and 400 mM β-mercaptoethanol] was added, followed by 10 min incubation at 95 °C prior to separation of lysates.

MARV infection assays. The Musoke strain of MARV was propagated in Vero cells and purified as described previously (Funke et al., 1995). Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FCS, 2 mM l-glutamine (Gibco) and 50 IU penicillin/streptomycin solution

<table>
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<th>siRNA/primer</th>
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RT-PCR primers

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<td>OAS-1</td>
<td>5’-AGGTGTGAAATGGTGGCC-3’</td>
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<tr>
<td></td>
<td>3’-ACAACGGTACGGTACGAT-5’</td>
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Cells were grown in 37 °C under 5 % CO₂. Twenty-four hours before transfection, Vero cells were trypsinized and transferred to Cellstar six-well plates (Greiner). Transfections with the indicated amounts of siRNAs were carried out as described by Bitko & Barik (2001) with minor modifications. Cells were transfected with siRNA using Lipofectamine 2000 in Opti-MEM I. Medium was replaced 4 h post-transfection with DMEM supplemented with 5 % FCS; cells were incubated for an additional 4 h and then infected with MARV at an m.o.i. of approximately 1 p.f.u. per cell. At 1 h post-infection, cells were washed twice with DMEM and a second siRNA transfection was performed. Four hours later, cells were washed and DMEM supplemented with 10 % FCS was added. At 24 h post-infection, cells and supernatants were harvested.

To harvest the supernatant, cell-culture medium was centrifuged for 2 min at 10000 r.p.m. to remove cellular debris. Supernatant was then mixed with loading buffer [100 mM Tris/ HCl (pH 6.8), 4 % SDS, 20 % β-mercaptoethanol, 20 % glycerol and 0.2 % bromophenol blue], boiled for 10 min and subjected to Western blot analysis. No estimation of total protein concentration was performed due to the presence of cellular factors, such as albumin and FCS, in the supernatant.

To harvest cells, monolayers were washed with PBS, trypsinized and washed with DMEM followed by centrifugation and a second washing step with PBS. After removal of PBS, 100 µl cell lysis buffer [50 mM Tris/ HCl (pH 7.5), 150 mM NaCl, 1 % NP 40, 1 µg complete protease inhibitor (Roche) ml⁻¹, 2 U DNase (Epicenter Technology)] was added to cell pellets. Cell pellets were resuspended, incubated at 37 °C for 10 min, supplemented with loading buffer and boiled for 10 min. Protein concentrations were quantified by performing SDS-PAGE followed by Coomassie staining. The gel was digitalized by using a GelDoc 2000 system (Bio-Rad) and protein amounts were normalized and quantified by using TINA 2.09 (Raytest).

Western blotting. Cell lysates were separated by 12 % SDS-PAGE and blotted onto PVDF membranes (Immobilon P; Millipore). Blots were incubated overnight at 4 °C in TBS [20 mM Tris/ HCl (pH 7.5), 150 mM NaCl] supplemented with 3 % BSA (Biomol). After removing BSA/TBS, blocks were incubated with the following antibodies diluted in 0.1 % Tween 20 in TBS (TBST): rabbit anti-NC serum (diluted 1:10 000) for detection of NP and VP35 and guinea pig anti-MARV VP30 serum (diluted 1:10 000); for MARV-infected cells: mouse monoclonal anti-NP antibody (1:10 000), guinea pig anti-VP35 serum (1:5000), guinea pig anti-VP30 serum (1:10 000), purified rabbit anti-VP24 antibody (1:10), rabbit anti-GP serum (1:2500) and mouse monoclonal anti-VP40 antibody (1:500). After 1 h incubation at room temperature with gentle shaking, blocks were washed with TBST and incubated with the appropriate secondary antibody (dilution 1:10 000 in TBST) coupled to horseradish peroxidase (Sigma.). Similarly, α-tubulin was detected with a 1:5000 dilution of mouse anti-α-tubulin IgG (Sigma) and reported with 1:10 000 diluted rabbit anti-mouse IgG coupled to horseradish peroxidase (Sigma). After washing with TBST, blots were treated with the Western Lightning Luminescence system (Perkin Elmer) according to the manufacturer’s instructions and exposed to Hyperfilm (Amersham Biosciences).

RT-PCR. Primer sequences are shown in Table 1. The human PKR gene was amplified with primers corresponding to the sequence (GenBank/EMBL accession no. U50648.1) from nt 632 to 807. The OAS-1 gene was amplified with primers corresponding to the sequence (accession no. NM_016816.1) from nt 203 to 272.

Cells were transfected with the plasmids encoding the nucleocapsid proteins and increasing amounts of siRNAs as described above, and total RNA was isolated at 24 h post-transfection with a Qiagen RNeasy Mini kit according to the manufacturer’s instructions. RNA was quantified by measuring A260 and diluted in sterile deionized water. Various amounts of RNA, ranging from 80 ng to 80 µg, were added to wells of a 96-well optical reaction plate (Applied Biosystems). Qiangen QuantiTect SYBR green RT-PCR kits were used, according to the manufacturer’s specifications, to perform RT-PCR amplification, which was monitored with an Applied Biosystems 7900HT sequence detection system. Initially, samples were heated to 50 °C for 30 min and 95 °C for 15 min, then 45 cycles of 94 °C for 20 s, 60 °C for 40 s and 72 °C for 40 s provided amplification and one cycle of 94 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s provided dissociation data. Data were analyzed with the SDS 2.0 program (Applied Biosystems) and reported as the number of cycles required to give a fluorescent signal at a particular threshold. The cycle number was then converted to relative RNA amounts based on linear graphic analysis.

RESULTS

siRNA decreases vector-based viral protein production

Due to their central role in the viral life cycle, we chose to target three of the nucleocapsid-protein transcripts, NP, VP30 and VP35, for destruction. Selection of siRNA sequences was made following the ‘Tuschl rules’ [http://www.rockefeller.edu/labheads/tuschl/sirna.html (Table 1)]. Plasmids (250 ng) encoding the MARV proteins were co-transfected with siRNA into HeLa cells. Forty-eight hours after transfection, cell lysates were collected and Western blotting was performed to detect MARV nucleocapsid proteins. A definite siRNA-dependent reduction of all three viral proteins in the lysates can be seen in Fig. 1. Additionally, probing of blots for the cellular protein α-tubulin resulted in no detectable reduction of this constitutively expressed cellular protein, indicating that reduction of viral proteins was not due to a general decrease in proteins present in the cell lysates.

To determine whether the presence of a non-virus-specific siRNA molecule could decrease vector-based protein production, siRNAs directed against a nuclear protein, lamin A/C, and a cellular transmembrane protein, CD44, were co-transfected with plasmids encoding the MARV proteins. No effect on viral protein production could be detected, suggesting that the reduction in viral protein seen in Fig. 1 required siRNA molecules that were targeted specifically to the viral transcript sequences.

siRNA does not increase transcription related to the interferon response

Introduction of dsRNA into cells can result in initiation of antiviral activities, including increased transcription of the interferon response-associated PKR and OAS-1 genes (Der et al., 1998). To determine whether siRNA-induced reduction of MARV proteins requires activity associated with the interferon response, we repeated co-transfection experiments with siRNA corresponding to the MARV NP sequence and collected total RNA from HeLa cell lysates 24 h post-transfection. Addition of poly(I:C), a synthetic construct similar to dsRNA, to cell medium served as a
positive control for elevated PKR and OAS-1 transcription. RT-PCR was utilized to detect changes in PKR and OAS-1 transcript levels in cell lysates. The highest concentration of MARV NP siRNA did result in an increase in both transcripts (Fig. 2). However, no significant difference in either PKR or OAS-1 transcript levels could be detected with siRNA concentrations that were high enough to effectively decrease plasmid-based production of MARV nucleoproteins (Fig. 1). This result suggests that RNAi, and not interferon-associated defences, are responsible for reduced levels of MARV proteins.

siRNA decreases viral protein production

As the chosen siRNAs effectively reduced plasmid-based production of MARV proteins and did not seem to trigger antiviral activity, the next question was whether these siRNAs would be able to specifically reduce proteins that are produced during MARV infection. However, HeLa CCL-2 cells were not susceptible to MARV infection (data not shown). Thus, we decided to use Vero cells, which have

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**Fig. 1.** RNAi-based reduction of plasmid-based Marburg viral protein expression in HeLa cells. HeLa cell monolayers were co-transfected with 250 ng plasmid expressing MARV nucleocapsid proteins and various amounts of siRNA molecules. At 48 h post-infection, lysates were collected and Western blotting was performed to detect viral nucleocapsid proteins and the cellular protein α-tubulin. Total protein concentration of lysates was determined with a standard Bradford assay and equal amounts of protein were loaded onto gels for SDS-PAGE. Lanes: 1, 250 ng empty plasmid; 2, 250 ng plasmid expressing the respective MARV nucleocapsid protein; 3, MARV plasmid+62.5 nM siRNA; 4, MARV plasmid+125 nM siRNA; 5, MARV plasmid+125 nM lamin A/C siRNA; 6, MARV plasmid+125 nM CD44 siRNA. Similar results were obtained in four separate experiments. HeLa cells were transfected with siRNA directed against (a) NP, (b) VP30 or (c) VP35.

**Fig. 2.** Levels of siRNA that are effective at reducing MARV nucleocapsid proteins do not increase interferon-associated PKR and OAS-1 transcripts. (a) OAS-1 transcript levels; (b) PKR transcript levels. Co-transfection assays were performed as described for Fig. 1 and total RNA was isolated at 24 h. Transcript levels of OAS-1 and PKR were determined by RT-PCR and reported as relative amounts of RNA. Lanes: 1, no vector, no siRNA; 2, 62.5 nM siRNA; 3, 125 nM siRNA; 4, NP vector; 5, NP vector and 62.5 nM siRNA; 6, NP vector and 125 nM siRNA; 7, poly(I:C) (166 ng ml⁻¹). The above data are reported as means ± SD of three separate analyses.
frequently been described to be susceptible to MARV. In a pilot experiment, we again tested the amount of siRNA that was necessary to silence transient expression of NP, VP35 and VP30. In Fig. 3, it is shown that, like in HeLa cells, the expression level of all three proteins could be diminished by the specific siRNA, but not by the control siRNA (siX, Fig. 3a–c). The expression level of cellular proteins was not changed by transfection of either specific or non-specific siRNA (siX), which has no homology to cellular or MARV genome targets (Fig. 3a–c, α-tubulin, lamin A/C).

We then transfected each siRNA into Vero cells prior to infection. Immediately after infection, cells were transfected a second time with the same amount of siRNA. At 24 h post-infection, cells were harvested and the amount of intracellular nucleocapsid and control cellular proteins was monitored by immunoblot analysis. A reduction in each targeted MARV protein was seen in MARV-infected cells treated with siRNA in comparison to cells left untreated (Fig. 4). Transfection of the non-specific siRNA siX had no effect on the expression level of any of the three MARV proteins, indicating that this reduction required specific targeting. Additionally, levels of the cellular proteins lamin A/C and α-tubulin were unchanged, indicating that siRNA-induced reduction of nucleocapsid proteins during MARV infection is not the result of a decrease in total protein production.

**siRNA-decreased nucleocapsid-protein levels result in decreased production of other viral proteins**

As the targeted nucleocapsid proteins are involved in virus replication, it is likely that reduction of one of the nucleoproteins will have an effect on production of non-targeted viral proteins. To investigate this possibility, Vero cells were treated with siRNA, infected with MARV and the presence of viral proteins NP, VP35, VP30, VP24 and VP40 in cell lysates was detected by Western blotting.

Treatment of MARV-infected cells with siRNA homologous in sequence to the MARV nucleocapsid protein VP35 (Fig. 5a, lane 3) showed only a slight effect on other viral proteins, in comparison to untreated infection and treatment with non-specific siRNA (Fig. 5a, lanes 2 and 6). However, treatment with siRNA homologous to MARV VP30 or NP led to a definite decrease in detection of all viral proteins tested (Fig. 5a, lanes 4 and 5). As no change in detection of tubulin and lamin A/C could be observed, this reduction in viral proteins is probably a result of a specific targeting of nucleocapsid protein and non-targeted reduction of other MARV proteins, due to disruption of the virus replication cycle.

**Reduced release of MARV virions from siRNA-treated cells**

This significant reduction in MARV protein production should result in decreased production of MARV progeny. MARV-infection assays were repeated and the amount of virus material released into the culture medium was detected by Western blot analysis. As NP is the most abundant component of the MARV virion, its release should best serve as an indication of virus production. Shown in Fig. 5(b), MARV infection led to the release of viral particles, as monitored by the amount of NP in cell media. Treatment of cells with an siRNA that was not...
homologous to the MARV genome, siX, had no effect on the release of NP from cells. In contrast, the amount of released NP was decreased dramatically when cells were treated with siRNA homologous to the MARV genes NP and VP30. Even treatment with the VP35-specific siRNA, which had only a slight effect on the intracellular levels of other MARV proteins (Fig. 5a), led to a reduction in released virions.

**DISCUSSION**

Here, we report the first use of RNAi to decrease MARV nucleocapsid proteins produced alone via plasmid transfection and in the natural viral-infection environment. Additionally, induction of the interferon response did not seem to play a role in the specific silencing of nucleocapsid proteins.

Whether RNAi is employed in natural antiviral defence is unknown. However, viral proteins can prevent RNAi, suggesting viral responses to negative pressure from RNAi...
in nature (Kubota et al., 2003; Reed et al., 2003; Thomas et al., 2003). It is possible that RNAi may involve portions of a natural antiviral defence programme that is at present poorly understood. Indeed, microarray analyses indicate that siRNA molecules can affect transcription levels of genes that are associated with the interferon response, such as PKR and OAS-1 (Bridge et al., 2003; Sledz et al., 2003). These reports, and the close association of virus replication with the interferon response, compelled us to address this issue. High concentrations of siRNA did elevate transcription of both PKR and OAS-1, agreeing with a report where increased levels of siRNA resulted in a dose-dependent increase in interferon-response transcription (Sledz et al., 2003); this titration effect could result in engagement of antiviral activities, clouding experimental conclusions. However, neither PKR nor OAS-1 transcript levels were elevated in the presence of siRNA concentrations that were shown to effectively reduce MARV replication and production, suggesting, at least, that the dsRNA-detection arm of the interferon response was not activated in our experiments.

Reducing NP protein levels in MARV-infected cells by RNAi resulted in a concomitant decrease in all viral proteins tested and in viral particles released from infected cells. This finding emphasized the previously reported central role of NP in virus replication and transcription (Mühlberger et al., 1998). VP35 has previously been shown to act as a cofactor to the MARV RNA-dependent RNA polymerase L and to be essential for viral transcription and replication (Mühlberger et al., 1998). Surprisingly, reduction of VP35 did not lead to a major reduction of other viral proteins. However, a significant reduction in the release of viral particles could be observed when intracellular VP35 levels were decreased. This suggested that VP35 has a secondary role as a determinant of viral assembly, probably at the level of nucleocapsid formation. We hypothesize that the reduced level of VP35 present in siRNA-treated cells was still high enough for its role as a polymerase cofactor as, most probably, only minute amounts were needed. However, this amount may not have been sufficient for maturation or assembly of nucleocapsids into viral progeny.

The function of VP30 in the MARV life cycle is currently unknown. The closely related Ebola virus (EBOV) VP30 has been shown to be an essential activator of EBOV-specific transcription (Modrof et al., 2002, 2003; Weik et al., 2002). Interestingly, although MARV VP30 was able to partially replace EBOV VP30 in transcription activation, MARV transcription was independent of VP30 in studies employing filovirus minigenome systems (Mühlberger et al., 1998, 1999). However, RNAi-based reduction of VP30 during MARV infection resulted in a strong reduction of the expression of other viral proteins and release of progeny virus. This result suggests that MARV VP30 does, indeed, play a role in either viral transcription or replication that may not have been detected in previous studies employing the minigenome system (Mühlberger et al., 1998).

Hopefully, further use of RNAi will provide more detailed observations on MARV-replication strategies.

The fact that RNAi can be employed to reduce virus replication begs the question of whether this technology can be employed effectively against viral disease. In studies using a viral-vector genome, McCaffrey et al. (2003) have shown that plasmid-based RNAi can decrease hepatitis B viral loads in a murine infection model. As such plasmid-based production of siRNA-like molecules is likely to provide only transient inhibition of virus replication, RNAi-based ‘vaccines’ seem to be out of reach at present. However, such a time frame could allow for a therapeutic post-infection response or give the infected host enough time to develop, for example, specific neutralizing antibodies that cannot be detected in fatal cases of filovirus infection (Baize et al., 1999). We feel that the results presented here are promising enough to initiate studies utilizing RNAi to reduce viral loads in vivo and possibly influence the outcome of viral infection.

ACKNOWLEDGEMENTS

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