The adenovirus VA RNA-derived miRNAs are not essential for lytic virus growth in tissue culture cells

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ABSTRACT

At late times during a lytic infection human adenovirus type 5 produces ~10⁸ copies per cell of virus-associated RNA I (VA RNAI). This short highly structured RNA polymerase III transcript has previously been shown to be essential for lytic virus growth. A fraction of VA RNAI is processed by Dicer into small RNAs, so-called mivaRNAs, which are efficiently incorporated into the RNA-induced silencing complex. Here, we constructed recombinant adenoviruses with mutations in the seed sequence of both the 5'- and the 3'-strand of the mivaRNAI duplex. The results showed that late viral protein synthesis, as well as new virus progeny formation, was essentially unaffected by the seed sequence mutations under lytic replicative conditions in HeLa or HEK293 cells. Collectively, our results suggest that either strand of the mivaRNAI duplex does not have target mRNA interactions that are critical for the establishment of virus growth under lytic conditions. Further, by depletion of protein kinase R (PKR) in HEK293 cells, we show that the suppressive effect of VA RNAI on the interferon-induced PKR pathway is most critical for late gene expression.

INTRODUCTION

Human adenoviruses undergo two types of infections: a rapid lytic infection and a slower persistent and/or latent infection. The lytic infection can be efficiently studied in cell lines, whereas a reproducible model system is still not well established for the persistent/latent infections. Here, we have analyzed the significance of the adenovirus-derived small RNAs for an efficient lytic virus infection.

Dependent on serotype, human adenoviruses encode for one or two virus-associated RNA (VA RNA) genes that are highly structured, low molecular weight (~160 nt), regulatory RNAs. Adenovirus 5 (Ad5) is the best-studied serotype and encodes two homologous VA RNA genes, VA RNAI and VA RNAII. VA RNAI has previously been shown to be non-essential for lytic growth (1). In contrast, the VA RNAII gene, which is expressed in ~10⁵ copies/infected cell (20-fold more compared with VA RNAI), is required to establish an efficient translation in late virus-infected cells (1). VA RNAI binds to the interferon inducible protein kinase R (PKR) thereby preventing it from becoming functionally activated by virus-derived dsRNA produced during the viral replication cycle (2). Activated PKR is designed to shut off translation in virus-infected cells by phosphorylating the translation initiation factor eIF-2α (3).

As many virus-encoded gene products, the VA RNAs seem to have more than one function. Thus, in addition to the PKR inhibitory activity of VA RNAI, both VA RNAI and VA RNAII have been shown to be processed by Dicer into small RNAs (so called mivaRNAs) that are incorporated into the RNA-induced silencing complex (RISC) and may play an important role in suppressing RNA interference (RNAi)/microRNA (miRNA) regulated pathways during virus infection (4–7). The VA RNAs are processed by Dicer at the terminal stem generating a 5' and a 3' mivaRNA strand. The strand incorporation of the mivaRNA duplex is highly asymmetric during a lytic infection with the 3'-strand being >200-fold overrepresented (8). Also, it has been suggested that VA RNAI suppresses the miRNA pathway in adenovirus-infected cells by inhibiting the export of premiRNAs and the Dicer mRNA to the cytoplasm by saturating the Exportin-5-dependent export pathway (6,9). Although only a few per cent of the VA RNAs are cleaved to mivaRNAs, the impact on RISC biology is most likely massive because the majority of the small...
RNAs associated with RISC have been shown to be VA RNA derived in late wild-type virus-infected cells (10). However, it is still unclear how the VA RNAs affect the cellular silencing machinery and whether this is beneficial for the virus infection.

It was recently shown that the mivaRNAs have the potential to regulate gene expression (11). By a microarray approach, it was shown that VA RNA expression results in a down- and upregulation of several hundred cellular mRNAs. As mRNA targets are selected based on the complementarity between a relatively short seed sequence in the miRNA (nucleotides: 2–8) and the 3' untranslated region in an mRNA (12,13), it is not surprising that mivaRNAs bound to RISC can have a regulatory effect on certain transcripts (11). However, one key question is whether these mivaRNA and mRNA interactions are important for lytic virus growth.

To test whether the small RNAs derived from VA RNAI have target mRNA interactions that are critical for lytic virus growth, we constructed recombinant adenoviruses encoding a single VA RNAI gene with 5' and 3' seed sequence mutations. Our results show that the mutated VA RNAI genes had the same expression profile as the wild-type VA RNAI gene, and that they were processed into mivaRNAs that were efficiently incorporated into RISC. Most importantly, mutation of the seed sequence in the 5' or 3'-mivaRNAs did not impair lytic virus growth in HEK293 or HeLa cells. Further, we present evidence that the mivaRNAs are not required for lytic infection. Using an siRNA knockdown approach of PKR and Dicer, we further present evidence that the suppressive effect of VA RNAI on the antiviral effect of the interferon-induced PKR enzyme is most critical for lytic virus growth in HEK293 cells.

MATERIALS AND METHODS

Plasmid and virus construction

To mutate the endogenous VA RNA genes in the pAdEasy-1 plasmid, a BstXI fragment (base pairs: 10035–14289) spanning the VA RNA region was subcloned into pUC18 (creating pUC18-VA). Overlap extension PCR reactions replacing the B-box of VA RNAI (primer sequences 5'-CGCGACTGCG-3' and 5'-AGTCGCGGGAAAGCTTTCGGACCGG-3') and VA RNAII (primer sequences 5'-CCGGACTCAACCCTTGGA-3' and 5'-GACGGCCGGATACGGAAGCTTCGTC-3') were carried out, and the PCR products were reintroduced into pUC18-VA using unique restriction sites creating pUC18–VA RNAI-5' and pUC18–VA RNAII-3'. The mutated VA RNAI and VA RNAII genes were reintroduced into pAdEasy-1 backbone plasmid by homologous recombination in Escherichia coli BJ5183. The constructs were verified by analysis of diagnostic HindIII restriction enzyme sites that replaced the B-box and by sequencing of the mutated regions. Thereafter the VAI wt, VAI 3'-mut and VAI 5'-mut genes were chemically synthesized (GenScript), cloned into plasmid pShuttle (14) and used to reconstruct viruses pAd-VAI wt, pAd-VAI 3'-mut and pAd-VAI 5'-mut, respectively. As a control, we also reconstructed a virus of the pShuttle-cytomegalovirus (CMV) plasmid (14), encoding for the GFP protein under the transcriptional control of the CMV promoter (pAd-ΔVA-GFP). Further details about the cloning strategies are available on request. The details about generation and amplification of the recombinant adenoviruses are described elsewhere (15).

Cell culture and virus infection

Viruses used in this study were Ad5, the VA RNAI/VA RNAII double-mutant virus dl720 (16) and the recombinant adenoviruses described earlier in the text. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen), 1% penicillin/streptomycin (PST) at 37°C in 5% CO2. To infect mammalian cells, the virus stock was thawed and mixed briefly. The required volume (calculated from desired number of virus particles per cell and the titer of the virus stock) was diluted in DMEM without serum. The medium was removed from the plate, and the diluted virus inoculum was added, followed by incubation for 1 h in a CO2 incubator (37°C, 7% CO2). After the 1 h incubation, the medium was removed, and DMEM, supplemented with 10% FCS and 1% PST, was added, and plates were returned back to the CO2 incubator.

siRNA knockdown

HEK293 cells were grown in DMEM supplemented with 10% FCS. Transfection of siRNA was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. To target PKR and Dicer, a pool of two siRNAs was used: PKR, 5'-GACCAGAAAGACU UACGUAUAtt-3' and 5'-GGUGAAGGUAGUACAAAG Att-3'; Dicer, 5'-UGCUGAAGCAGCUCCUGAtt-3' and 5'-UUUGUUCGAGGGCCUAtt-3'. To detect specific effects of siRNAs, a scrambled ON-TARGET plus non-targeting siRNA (Thermo Scientific) was used as a control.

RNA extraction and RT–PCR

Cytoplasmic RNA was prepared by lysis with IsoB-NP-40 buffer [10 mM Tris–HCl (pH 7.9), 150 mM NaCl, 1.5 mM MgCl2 and 1% Nonidet P-40], followed by two rounds of phenol–chloroform–isoamylalcohol extraction and one extraction with chloroform–isoamylalcohol. Total RNA was extracted using the TRizol reagent (Sigma) according to the manufacturer’s instructions. The cDNA synthesis was performed using the SuperScript III Reverse Transcriptase kit according to the manufacturer’s instructions (Invitrogen). PCR was performed under the following cycling conditions: 94°C, 5 min, once; 94°C, 30 s, 56°C, 30 s, 72°C, 30 cycles and finally 72°C, 5 min, once. PCR products were examined by electrophoresis on a 1% agarose gel. The primers used were TIA-1, forward 5'-GG ACAGAGATGCCCAAAGACTC-3' and reverse, 5'-ACAA AGACATGGAATAATGATTGT-3'; GAPDH, forward 5'-CCGACATTCTTTTGTGCCTGC-3' and reverse, 5'-G ATCTCGCTCTGGAGATGG-3'.
RISC immunoprecipitation and northern blot analysis

Immunoprecipitation of the FLAG/HA Ago2 protein was done as described previously (17). For northern blot analysis, RNA was separated on a denaturing 12% polyacrylamide gel and transferred to a Hybond NX membrane (Amersham Biosciences), chemically cross-linked and hybridized as previously described (18). Hybridization probes were generated by 5'-end labeling of DNA oligonucleotides complementary to the 5' or 3'-strands of VA RNAI (wt, 3'-mut and 5'-mut) or to the 7SL RNA, which was used as a loading control (for probe nucleotide sequences see Supplementary Table S1). The oligonucleotides used to detect the wt, 3'-mut and 5'-mut VA RNAI sequences were mixed 1:1:1 and 5'-end labeled in the same reaction. Membranes were prehybridized in 5× Denhardt’s solution [6× Saline-Sodium Citrate (SSC) and 0.2% Sodium Dodecyl Sulfate (SDS)] for 2 h at 42°C. After overnight hybridization with the probe, the membrane was washed in 3× SSC and 0.5% SDS three times for 10 min at 42°C and once with 1× SSC, 0.5% SDS for 15 min at 42°C. Signals were detected by exposure of membranes to a PhosphorImager screen (Fuji) followed by signal analysis with the Image One software (BioRad).

Total protein extraction and western blotting analysis

Cells were collected and washed once with 1× phosphate-buffered saline (PBS). The cell pellets were resuspended in 320 μl of Radioimmunoprecipitation assay buffer (RIPA) [150 mM NaCl, 50 mM HEPES (pH 7.4), 0.5% sodium deoxycholate and 0.1% SDS] supplemented with 1 U/ml of benzonase (EMD Millipore) by vortexing and incubated for 1 h at 4°C. Thereafter, 40 μl of 10% SDS and 40 μl of 1 M Dithiotreitol (DTT) was added, and the samples were boiled for 5 min. Protein samples were separated on an AnyKD™ or a 4–20% gradient pre-cast gel (Bio Rad). Proteins were electro-transferred (Bio Rad blotting chamber) onto an Immobilon-FL western blot nitrocellulose membrane (Millipore) at 200 mA for 2–6 h, using Towbin transfer buffer (25 mM Tris and 192 mM glycine). The membrane was blocked with Odyssey blocking buffer (Li-COR) for 1 h at 4°C and thereafter incubated with the primary antibody, diluted in the blocking buffer, overnight at 4°C. The membrane was washed four times for 5 min in 1× PBS with 0.01% Tween-20 followed by incubation with the fluorescent secondary antibodies (IRDye®. LI-COR) diluted in the blocking buffer, for 1 h at room temperature. The membrane was washed as described earlier in the text and finally rinsed in 1× PBS and scanned with the Odyssey scanner (Li-COR). The following primary antibodies were used in the study: anti-actin (Santa Cruz), anti-Ad5 (Abcam), anti-pKR (SantaCruz), and anti-Dicer (Abcam).

35S-methionine/cysteine metabolic labeling

Before cell pulse labeling, the medium was removed, and the cells were washed once in 1× PBS and incubated in methionine/cysteine-free DMEM (supplemented with 10% FCS, 1% PEST and 2% glutamine) for 1 h. After the starvation period, the medium was removed and fresh methionine/cysteine-free DMEM supplemented with 35S Protein Labeling Mix (50 μCi per 2 ml of media) was added, and the cells incubated for 2 h in a CO2 incubator (37°C, 7% CO2). The cells were harvested, and total protein extracts were prepared as aforementioned.

MTS assay

Human embryonic retina 911 cells (19) were seeded in 96-well plates (5000 cells/well) in DMEM supplemented with 5% FCS and 1% PEST. Eighteen hours later, cells were infected with 5-fold serially diluted aliquots of the indicated viruses starting at 1000 fluorescence forming units (FFU)/cell. Cell viability (EC50 value—concentration of viruses that cause a 50% reduction in cell viability) was assayed at 4 and 6 days post-infection (dpi) using the Non-radioactive cell proliferation assay (MTS assay) (Promega), according to the manufacturer’s instructions. Results are presented as the mean of three experiments.

RESULTS

Construction of an adenoviral vector backbone devoid of VA RNA expression

The VA RNAs are transcribed by RNA polymerase III from internal promoters consisting of two essential regulatory elements, the A-box and the B-box (20). To inactivate the expression of the endogenous VA RNA genes, we mutated the B-box in both the VA RNAI and VA RNAII promoters (Figure 1B). By this strategy, we generated a new AdEasy vector backbone devoid of functional VA RNAI and VA RNAII genes (pAd-ΔVA). To generate the control virus, Ad-ΔVA-GFP, expressing a GFP reporter gene under the transcriptional control of a CMV promoter, plasmid pShuttle-CMV (14) was reconstituted to a virus by homologous recombination with the modified Ad-ΔVA genome depicted in Figure 1A. To characterize VA RNA expression, cytoplasmic RNA was prepared from HEK293 cells transfected with plasmids expressing either VA RNAI or VA RNAII or infected with the Ad-ΔVA-GFP virus and analyzed by northern blotting using a DNA probe detecting both VA RNAs. Although the VA RNAs are identical in length, VA RNAII seems to have more compact structure migrating significantly faster than VA RNAI on a denaturing polyacrylamide gel (Figure 1C, lanes 1 and 2) (22). The absence of expression of both VA RNAs in the Ad-ΔVA-GFP virus-infected cells could, therefore, independently be confirmed (Figure 1C, lane 3). As expected from previous results (23), VA RNAI has the strongest promoter and is the predominant VA RNA species expressed in Ad-infected cells (Figure 1C, lane 4).

Construction of recombinant adenoviruses with mutated mivRNAI seed sequences

To be able to study the function of the VA RNAI-derived 5'- and 3'-mRNAI sequences on virus growth, the GFP cassette in Ad-ΔVA-GFP was replaced with the wild-type VA RNAI gene or VA RNAI genes with 5'- and 3'-seed sequence
mutations (Figure 2), generating recombinant viruses Ad-VAI wt, Ad-VAI 5'-mut and Ad-VAI 3'-mut, respectively. The miRNA seed sequence (nucleotides 2–8) is the key element mediating pairing between an miRNA and a target mRNA (12). The seed mutations introduced here were selected such that they would not interfere with the A-box of the internal VA RNAI promoter but destroy seed pairing between the 5'- and 3'-mivaRNAI and their hypothetical target mRNA(s). It should be noted that VA RNAI has two start sites (24,25). A minor VA RNAI(A) start and a major VA RNAI(G) start that initiates transcription three nucleotides downstream of the A start. We have previously shown that the 5'-strand of the VA RNAI(A) start mivaRNA generates RISC complexes with significantly higher cleavage activity compared with the 5'-strand of the VA RNAI(G) start mivaRNA (8). The efficiency of RISC loading is to a large extent believed to correlate with the thermodynamic stability at the ends of a siRNA duplex (27,28) with the less stable 5'-end being preferred in RISC assembly. As we introduced mutations into the seed sequences of VA RNAI, we examined whether these mutations also affected strand selection during RISC loading. For this experiment, we infected an HEK293 stable cell line expressing a FLAG/HA-tagged Ago2 protein (293-Flag-Ago2) (10), with wild-type Ad5 or the recombinant VAI

Ad-ΔVA-GFP–infected cells showed, as expected, a complete lack of VA RNAI expression (lane 6). In contrast, Ad-VAI 3'-mut– (lane 4) and Ad-VAI 5'-mut (lane 5)–infected cells showed similar levels of VA RNAI expression compared with Ad-VAI wt–infected cells (lane 3). Taken together, these results demonstrate that introduction of the seed sequence mutations into the 5'- and 3'-strands of mivaRNAI did not adversely affect VA RNAI transcription. 

Mutating the 5'- and 3'-seed sequences in the mivaRNAI duplex results in a strand switch in RISC loading

We have previously shown that the processed mivaRNAI duplex show a highly asymmetric RISC loading with the 3'-strand of mivaRNAI incorporated into RISC with >200-fold higher efficiency compared with the 5'-strand (8). The efficiency of RISC loading is to a large extent believed to correlate with the thermodynamic stability at the ends of a siRNA duplex (27,28) with the less stable 5'-end being preferred in RISC assembly. As we introduced mutations into the seed sequences of VA RNAI, we examined whether these mutations also affected strand selection during RISC loading. For this experiment, we infected an HEK293 stable cell line expressing a FLAG/HA-tagged Ago2 protein (293-Flag-Ago2) (10), with wild-type Ad5 or the recombinant VAI

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**Figure 1.** Construction of a VA RNAI and VA RNAII B-box double mutant virus. (A) Schematic drawing showing a simplified transcription map of the Ad5 genome with the position of the early (open arrows) and late transcription units (filled arrows) indicated. The deletions in the E1, E3 and VA RNA regions are indicated by crosses [see (14) and later in the text]. The box-labeled GFP/VA indicates the position where the modified VA RNAI genes and the GFP reporter gene were inserted. (B) Expansion of the genome position encoding the VA RNA genes with the mutations introduced into the B-box of VA RNAI and VA RNAII indicated. Dashes indicate nucleotides deleted during the construction of the variant VA RNA genes, with a HindIII cleavage site replacing the box B position. (C) Representative northern blot of VA RNA expression in the parental AdEasy virus and the VA RNAI/VARNII-deleted virus, Ad-ΔVA-GFP. As a control, cytoplasmic RNA isolated from pVAI and pVAII (21)-transfected cells were also analyzed.
mutant viruses. After incubation, cytoplasmic S15 extracts were prepared, and Ago2 containing complexes were captured by immunoprecipitation with an anti-FLAG M2 agarose resin. The small RNA content was examined by northern blot analysis using 32P-labeled single-stranded DNA oligonucleotide probes detecting either the 3'- or 5'-mivaRNAI (Figure 3B). As expected from our previous results (10), the RISC assembly of the mivaRNAI duplex was highly asymmetric with the 3'-mivaRNAI being the major mivaRNAI associated with immunopurified RISC (Figure 3B, lane 2). A similar 3'-mivaRNAI preference was also observed in Ad-VAI wt-infected cells (Figure 3B, lane 3). Interestingly, in Ad-VAI 3'-mut-infected cells, strand selection was reversed with the 5'-mivaRNAI preferentially assembled into RISC (Figure 3B, lane 4). This switch in RISC loading was not entirely unexpected, as the seed mutations introduced in the VA RNAI 3'-mut caused an increase in the GC content at the 5'-end of 3'-mivaRNAI (Figure 2). As predicted from the mutations introduced, the VA RNAI

Figure 2. VA RNAI seed sequence mutations. Schematic drawing showing the secondary structure of VA RNAI with the 5'- and 3'-seed sequence mutations indicated in bold. The strand designation is illustrated for the VA RNAI wt transcript. The arrows indicate the position of the Dicer-processing site.
5'-mut showed an asymmetry in strand selection similar to the VA RNAI wt, with the 3'-mivaRNAI preferentially assembled into RISC (Figure 3B, lane 5).

**Mutation of the seed sequence in the 5'- or 3'-mivaRNAI does not perturb lytic adenovirus growth in HEK293 cells**

The fact that the VA RNAs are processed into small RNAs is an interesting observation that leads to the more important question whether the mivaRNAs have an essential function for lytic virus growth. By a microarray approach, it has been shown that several hundred cellular mRNAs are up- or downregulated in response to VA RNA overexpression (11). Among them, the T-cell-restricted intracellular antigen-1 (TIA-1) mRNA and protein expression was reported to be reduced by the 3'-mivaRNAI known as mivaRNAI-138 (11). As shown in Figure 4, the steady-state level of TIA-1 mRNA was reduced in cells infected with the VA RNAI wild-type virus at both 16 and 20 hour post-infection (hpi) (compare lane 1 with 2 and 7). Similarly TIA-1 mRNA expression was repressed in the 5'-seed sequence mutated virus (lanes 4 and 7). Interestingly, the mutations destroying the 3'-mivaRNAI-138 seed sequence failed to block TIA-1 mRNA accumulation (lanes 3 and 6). Taken together, these results suggest that mivaRNAI-138 has an miRNA function and is able to regulate cellular gene expression during a lytic infection. However, this does not necessarily prove that this miRNA function is required for the lytic life cycle of the virus. Therefore, to determine whether mutations in the mivaRNAI seed sequences have an impact on a lytic infection, HEK293 cells were infected with the Ad-VAI wt or the Ad-VAI seed sequence mutated viruses. After 20 hpi, the cells were 35S pulse-labeled for 2 h, proteins separated on an SDS–PAGE, and late viral protein synthesis visualized either by autoradiography (Figure 5A) or western blot analysis detecting adenovirus capsid proteins (Figure 5B). The results from both assays show that the synthesis rate of proteins (Figure 5A) and the steady-state level of late protein accumulation (Figure 5B) were essentially identical in Ad-VAI 3'-mut (lane 4), Ad-VAI-5'-mut (lane 5) and Ad-VAI wt (lane 3)-infected cells. In addition, the recombinant viruses all seemed to accumulate late proteins to the same extent as the Ad5 virus (lane 2). Similar results were observed in 911 cells (data not shown). Also, as expected from previous results (1), the absence of VA RNA expression effectively abolished synthesis and accumulation of late viral proteins (Ad-ΔVA-GFP; lane 6).

Although late protein synthesis seemed to be normal in the mutant virus-infected cells, we tested the possibility that the mutations in the seed sequences may perturb a subsequent and essential function for efficient virus production. By a microarray approach, it has been shown that several hundred cellular mRNAs are up- or downregulated in response to VA RNA overexpression (11). Among them, the T-cell-restricted intracellular antigen-1 (TIA-1) mRNA and protein expression was reported to be reduced by the 3'-mivaRNAI known as mivaRNAI-138 (11). As shown in Figure 4, the steady-state level of TIA-1 mRNA was reduced in cells infected with the VA RNAI wild-type virus at both 16 and 20 hour post-infection (hpi) (compare lane 1 with 2 and 7). Similarly TIA-1 mRNA expression was repressed in the 5'-seed sequence mutated virus (lanes 4 and 7). Interestingly, the mutations destroying the 3'-mivaRNAI-138 seed sequence failed to block TIA-1 mRNA accumulation (lanes 3 and 6). Taken together, these results suggest that mivaRNAI-138 has an miRNA function and is able to regulate cellular gene expression during a lytic infection. However, this does not necessarily prove that this miRNA function is required for the lytic life cycle of the virus. Therefore, to determine whether mutations in the mivaRNAI seed sequences have an impact on a lytic infection, HEK293 cells were infected with the Ad-VAI wt or the Ad-VAI seed sequence mutated viruses. After 20 hpi, the cells were 35S pulse-labeled for 2 h, proteins separated on an SDS–PAGE, and late viral protein synthesis visualized either by autoradiography (Figure 5A) or western blot analysis detecting adenovirus capsid proteins (Figure 5B). The results from both assays show that the synthesis rate of proteins (Figure 5A) and the steady-state level of late protein accumulation (Figure 5B) were essentially identical in Ad-VAI 3'-mut (lane 4), Ad-VAI-5'-mut (lane 5) and Ad-VAI wt (lane 3)-infected cells. In addition, the recombinant viruses all seemed to accumulate late proteins to the same extent as the Ad5 virus (lane 2). Similar results were observed in 911 cells (data not shown). Also, as expected from previous results (1), the absence of VA RNA expression effectively abolished synthesis and accumulation of late viral proteins (Ad-ΔVA-GFP; lane 6).
growth. For this experiment, 911 cells (19) were infected with serial dilutions of the VA RNA wild-type and seed sequence mutant viruses and the cytotoxic activity tested in a cell viability assay (MTS assay) at 4 and 6 days post-infection (Figure 6). As expected from earlier results, the Ad-ΔVA-GFP virus showed a 20-fold reduction in cytotoxicity at day 6 compared with Ad-VAI wt expressing the wild-type VA RNAI gene. Importantly, both Ad-VAI 3’-mut and Ad-VAI 5’-mut exhibited essentially the same cytotoxicity as the recombinant virus expressing the wild-type VA RNAI gene. The inhibition of the VA RNA-deleted virus was apparent at Day 4 with a 2- to 3-fold lower cytotoxicity compared with the VA RNAI wild-type and mutant expressing viruses. Taken together, our results suggest that the seed sequence interaction of the 5’- or the 3’-mivaRNAI is not essential for the establishment of an efficient lytic adenovirus infectious cycle in 911 cells.

The seed sequence mutated VA RNAI support adenovirus growth in HeLa cells

To determine whether the lack of a phenotype for the mivaRNAI seed sequence mutant viruses also could be observed in another standard human cell line used in adenovirus research, we tested the significance of the seed pairing interactions in a HeLa cell infection. However, as the recombinant viruses we constructed based on the AdEasy system that lacks most of the Ad5 E1 region, they cannot replicate in non-E1 expressing cell lines, like HeLa cells. To overcome this problem, we resorted to a trans-complementation assay. In this experiment, HeLa cells were co-infected with the seed sequence mutant viruses and the Ad5 mutant dl720, which has a wild-type E1 region but is defective in both VA RNAI and VA RNAII expression (16). The rationale for this experiment is that dl720 will provide the E1 functions, whereas the recombinant viruses will provide the VA RNAI function (wt or seed mutant). In this experiment, HeLa cells were infected with combinations of the viruses, and infected cells were pulse-labeled 46 hpi with 35S-methionine. Protein extracts and total cytoplasmic RNA were prepared and analyzed by SDS-PAGE and by northern blotting. As shown in Figure 7A, infection of HeLa cells with the recombinant viruses alone (lanes 4–7) did, as expected, not result in an inhibition of host cell gene expression or an induction of efficient late viral protein synthesis. In contrast, co-infection of dl720 and the

Figure 5. Mutating the 5’- or 3’-mivaRNAI seed sequence does not impair virus growth in HEK293 cells. (A) HEK293 cells were infected with the indicated viruses (5 FFU/cell) followed by a 35S-methionine pulse labeling at 24 hpi. Total protein extracts were separated on an SDS-PAGE, and protein synthesis was visualized by autoradiography. (B) Total late viral protein accumulation was also detected on the same samples by western blot analysis using an anti-Ad5 capsid antibody. Equal loading of protein samples was confirmed by western blot using an anti-actin antibody. The position of major viral proteins is indicated to the right of each panel.

Figure 6. Comparable cytotoxicity of VA RNAI wild-type and seed sequence mutant viruses. The indicated viruses were 5-fold serially diluted on 911 cells from a starting point of 1000 FFU/cell. At 4 and 6 days post-infection, MTS assay was performed and the EC50 value calculated. Shown is the fold increase in viral cytotoxicity compared with the VA RNA negative Ad-ΔVA-GFP virus. *P < 0.05, the data shown are the mean values of three independent experiments ± SEM.
recombinant viruses resulted in an efficient shut-down of host cell gene expression and an essentially complete rescue of late viral protein synthesis (compare lane 2 and lanes 8–10). The rescue of late viral protein synthesis requires VA RNAI expression (Figure 7A, lane 11) and correlates with a dramatic increase in VA RNAI accumulation (Figure 7B, lanes 8–10), most likely resulting from viral DNA replication causing an increase in the copy number of the VA RNA gene. Collectively, our results suggest that seed sequence interaction of the 5′ or the 3′ mivaRNAI is not essential for the establishment of an efficient lytic adenovirus infection in HeLa, 911 or HEK293 cells.

Depletion of PKR protein expression substitutes for VA RNAI expression during an adenovirus infection

VA RNAI has a well-characterized function as a suppressor of the interferon-induced PKR enzyme (29,30). The results presented earlier in the text strongly suggest that the mivaRNAs derived from VA RNAI do have the capacity to regulate cellular gene expression (Figure 4), but that this function is not essential to establish a lytic infection. Thus, we do not find evidence that regulation of natural target mRNAs by the mivaRNAIs is required for establishment of an efficient lytic infection. A recent report has suggested that knockdown of the Dicer enzyme is sufficient to fully rescue growth of the VA RNA double mutant virus dl720 (9).

To test whether the PKR or RNAi/miRNA pathways are most critical for the establishment of a lytic adenovirus infection, we used an siRNA approach to knockdown PKR or Dicer expression in VA RNA double mutant virus (dl720)-infected cells. In this experiment, HEK293 cells were pre-treated with siRNA for 24 or 48 h, followed by infection with mutant dl720 or Ad5 wild-type (5 FFU/cell). Cells were 35S-methionine pulse-labeled 22 hpi, and protein extracts were prepared and separated on an SDS-PAGE. Late viral protein synthesis was visualized by autoradiography. The position of major viral capsid proteins is indicated to the right of the panel. The efficiency of siRNA knockdown was confirmed by immunoblotting of cell extract with antibodies directed against actin, Dicer and PKR. Scr, scrambled siRNA.

Figure 8. Rescue of the dl720 (VA RNAI+/VA RNAII−) mutant phenotype by PKR knockdown. HEK293 cells were transfected with the indicated siRNAs for 24 h, followed by infection with mutant dl720 or Ad5 wild-type (5 FFU/cell). Cells were 35S-methionine pulse-labeled 22 hpi, and protein extracts were prepared and separated on an SDS-PAGE. Late viral protein synthesis was visualized by autoradiography. The position of major viral capsid proteins is indicated to the right of the panel. The efficiency of siRNA knockdown was confirmed by immunoblotting of cell extract with antibodies directed against actin, Dicer and PKR. Scr, scrambled siRNA.
multiplication. However, a knockdown of PKR almost completely rescued late viral protein synthesis (compare lanes 3 and 4). Collectively, these results suggest that, under conditions where no mivaRNAI are produced, the removal of PKR expression is sufficient to almost fully rescue viral late gene expression. The implication of these results for mivaRNAI function is further discussed later in the text.

**DISCUSSION**

In this study, we have analyzed the impact of the Ad5 VA RNAI-derived mivaRNAs for the lytic life cycle of adenovirus. Our results show that site-directed interference with the mivaRNAI seed sequence does not affect the lytic growth properties of the virus. To the best of our knowledge, this is the first report addressing the importance of the mivaRNAs for lytic adenovirus growth. In addition, we provide evidence that the main function of VA RNAI during a lytic infection is to block the negative effects of interferon signaling via the PKR pathway. In contrast, blocking pre-miRNA processing by knockdown of the Dicer enzyme did not have a major impact on the lytic growth potential of adenovirus in HEK293 cells. This result is in striking contrast to the recent report, suggesting that Dicer knockdown can rescue virus replication (9). However, we note that the authors used viral DNA replication as a measure for virus growth. This assay is probably not the best to use, as previous results have suggested that the VA RNAI phenotype is manifested at a step subsequent to viral DNA replication (1,31). However, it should be noted that our results do not demonstrate that the cellular miRNA pathway is of no significance for a lytic adenovirus infection. Since, miRNA/RISC complexes are stable in a cell with an estimated average miRNA half-life of almost 5 days (32). Therefore, a knockdown of Dicer for 24 or 48 h would not necessarily be expected to have a damaging effect on the pool of cellular miRNAs.

Adenovirus has developed several strategies to target the RNAi/miRNA pathways during infection. Available data point to a major role of the VA RNAs in these processes (4,8,10). Thus, the VA RNAs interfere with the RNAi/miRNA pathways both at the level of pre-miRNA processing and RISC assembly. The VA RNAs are produced in a high abundance and have a squelching effect on Dicer cleavage of exogenous substrates (4), and as a consequence, they also become a preferred substrate for RISC assembly.

Further, it has been shown that the terminal stem of VA RNAI binds to the Exportin-5 receptor (33). This binding reduces the efficacy of cellular pre-miRNA binding to Exportin-5 (6), a function that has been suggested to have an important role in virus replication (9). Here, we show that modulation of the terminal stem structure, by site-directed mutagenesis targeting the seed sequence, did not have a significant impact on the accumulation and processing of VA RNAI into mivaRNAs (Figure 3). Thus, VA RNAI expression and export are controlled in a seed-sequence–independent manner in adenovirus-infected cells.

Since the discovery of the adenovirus-encoded mivaRNAs, a main objective in this area of research has been to understand the functional significance of these viral miRNAs during the virus life cycle. Theoretically, several alternative mechanisms can be envisioned. For example, the mivaRNA may target cellular mRNAs and thereby regulate their stability and function. Indeed, several cellular mRNAs containing complementary sequences to the mivaRNA seed sequence have been identified (11). In fact, it has been demonstrated that the mivaRNAs have the capacity to regulate cellular gene expression. For example, the TIA-1 mRNA levels were reduced by overexpression of mivaRNAI-138 (11). The same report also confirmed the negative effect of VA RNA on TIA-1 mRNA expression during an adenovirus infection. However, the significance of these changes in TIA-1 expression for virus growth was not tested. Our results confirm the finding that mivaRNAI-138 has the capacity to reduce TIA-1 mRNA expression during a lytic infection. However, our results also clearly argue against the model that mivaRNA regulation of cellular gene expression is critical for establishment of an efficient lytic infection (Figures 5–7).

It is possible that the adenovirus-encoded mivaRNAs are important for virus multiplication by targeting viral mRNAs. Indeed, DNA virus-encoded miRNAs have been shown to control gene expression as exemplified by the simian vacuolating virus 40 (SV40)-encoded miRNA controlling T-antigen expression (34). However, under our experimental conditions, one would have expected to detect differences in adenovirus growth when using the seed-sequence–mutated viruses. Our data point to a non-essential role of the mivaRNA seed sequence mRNA interactions for establishment of a lytic adenovirus infection (Figures 5 and 6). Therefore, it also seems unlikely that the mivaRNAs remodel viral gene expression by targeting complementary viral RNA molecules. Rather, the main role of mivaRNAs during a lytic infection might be to saturate RISC and thereby transform the host cellular miRNA composition in RISC during an infection. Such a function might be important to inhibit a potential targeting of adenoviral mRNAs by cellular miRNAs with an antiviral activity. There are several examples of cellular miRNAs that have an antiviral activity. For example, miR-27 has been shown to inhibit murine cytomegalovirus replication (35). Also, a recent report has demonstrated that miR-214, which represses E1A protein expression, hampers adenovirus growth (36).

It seems likely that the VA RNAI seed sequence mutations did not affect adenovirus replication because of the rapid progression of the adenovirus lytic life cycle. It should be noted that the read-out of the RNAI pathway, such as reduced accumulation of proteins, takes a considerable time to manifest. Also, the activity of individual RNAI protein components can be altered depending on prolonged cell growth conditions, like senescence (37). In our experiments, we studied the role of mivaRNA mutant viruses during lytic virus life cycle, which is completed within two days. Thus, it seems likely that the time-span of the experimental approach is too short to detect significant changes in virus replication because of changes in the
cellular miRNA pool. Further, we show (Figure 6) that the production of infectious virus particles was essentially identical between the wild-type and seed sequence mutant viruses. This observation clearly confirms that the VA RNAi mutants can completely fulfill all wild-type VA RNAi functions during a lytic virus infection.

Interestingly, adenovirus can also infect lymphoid cells causing what seem to be persistent/latent infections (38). Thus, it is possible that the mivaRNAs may have a significant function under these types of prolonged infections. This hypothesis is supported by the observation that, for example, virus-encoded miRNAs have been shown to have distinct functions during the latent infection cycle as shown in the case in Kaposi’s sarcoma-associated herpesvirus (39). Therefore, we speculate that the mivaRNAs may have a significant impact on adenovirus growth during the persistent/latent infectious cycle, something that is not observable during lytic growth conditions. It will be interesting to explore the function of our mivaRNA-mutated viruses during conditions where the virus causes persistent/latent infections.

VA RNAI is a multifunctional molecule targeting both the anti-viral interferon pathway, as well as the RNAi pathway. Our data suggest that knockdown of the cellular PKR pathway almost completely alleviated the requirement of VA RNAI, whereas Dicer knockdown had no detectable effect on viral late protein expression (Figure 8). Moreover, a double knockdown of both PKR and Dicer did not result in a more efficient rescue of late viral protein expression compared with PKR knockdown alone (data not shown), suggesting, under these conditions in HEK293 cells, the RNAi/miRNA pathway did not have a significant inhibitory effect on virus replication. These results are in agreement with the previous observation that in the absence of VA RNAI late viral miRNAs are inefficiently translated, although they accumulate at similar levels as in a wild-type infection (1).

The observation that mutations of the mivaRNAI 5’- or 3’-seed sequence produce a viable virus may have a major impact on adenoviral vector design by offering the possibility to replace the mivaRNAs with a therapeutic siRNA. Further, it might be possible to manipulate the thermodynamic stability of VA RNAI in a way that will significantly impair the loading of both strands of mivaRNAI into RISC. Such a strategy might reduce the off-target effects, these viral miRNAs might have.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Table 1.

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