INTRODUCTION

MicroRNAs (miRNAs) are non-coding RNAs of around 21–23 nt that repress cognate miRNAs, often referred to as target miRNAs. They are transcribed as long hairpin-containing precursors, which are further processed into a stem–loop structure by the RNase III Drosha and, after export to the cytoplasm, are processed by a second RNase III, called Dicer (Bartel, 2004; He & Hannon, 2004; Lee et al., 2002). One strand of the resulting short duplex is then incorporated into a member of the Argonaute (Ago) protein family, the functional component within the RNA-induced silencing complex (RISC) (Bartel, 2004; Du & Zamore, 2005; Meister & Tuschl, 2004). Once loaded, the mature miRNA can remain stable with a mean half-life of several days (Gantier et al., 2011), whilst the second strand is rapidly degraded. Functionally, miRNAs act at the post-transcriptional level, thus adding another possible means of regulating gene expression for a number of biological processes including the cell cycle, metabolism and development (Biggar & Storey, 2011; Bueno & Malumbres, 2011; Sayed & Abdellatif, 2011).

Several viruses, the majority from the family Herpesviridae, encode their own miRNAs and use the host-cell miRNA pathway both to regulate their own life cycle, (e.g. the switch from latent to lytic infection) and to manipulate the host system in a non-immunogenic manner (Plaisance-Bonstaff & Renne, 2011; Tuddenham & Pfeffer, 2011). Host miRNAs can also be co-opted for virus replication, such as miR-122 which activates hepatitis virus C translation (Jopling et al., 2005). Conversely, host miRNAs can have antiviral functions by targeting the viral genome or downregulating essential components for infection. Vesicular stomatitis virus is directly targeted by miR-24 and miR-93 (Otsuka et al., 2007), and RIG-I-inducible miR-23b inhibits infections by minor-group rhinoviruses through downregulation of the receptor VLDLR (Ouda et al., 2011). In turn, miRNAs, which may have antiviral effects, can be downregulated by viral factors, e.g. miR-100 and miR-101, which regulate mTOR signalling components and are downregulated by human cytomegalovirus (HCMV) infection (Wang et al., 2008).

It has previously been shown that murine cytomegalovirus (MCMV) encodes 18 pre-miRNAs that are highly expressed during lytic infection using a small-RNA cloning
approach (Buck et al., 2007; Dölkken et al., 2007). Analysis of cellular miRNAs revealed only slight changes, indicating that even high levels of viral miRNA have no global impact on the host miRNA system. The only exception found was miR-27a, which was rapidly downregulated post-transcriptionally and was shown to have antiviral properties (Buck et al., 2010). Interestingly, miR-27a is also targeted for degradation by the herpesvirus saimiri non-coding RNAs HSUR 1 and 2 (Cazalla et al., 2010).

In this study, we wanted to investigate further the impact of MCMV infection on the mouse miRNA system. To this end, we constructed small-RNA libraries suitable for deep sequencing of both mock- and MCMV-infected cells, as well as after RISC immunoprecipitation (IP), to check for expression and also loading of both viral and mouse miRNAs into the functional component. We found that viral miRNAs only comprised ~13 % of all miRNAs late after infection. A novel MCMV mature miRNA, m01-1-3p, was detected, indicating that our sequencing covered the small-RNA profile at least as comprehensively as previous reports. After RISC IP, the viral miRNA component was not increased, suggesting that viral miRNAs were not preferentially loaded. Whilst most cellular miRNAs remain unchanged following MCMV infection, we could confirm downregulation of miR-27a. In addition, we found two more host miRNAs that are influenced by virus infection, miR-26a, which was moderately downregulated in fibroblasts but not in epithelial cells, and miR-7a, which was strongly upregulated in both cell types. Neither virus entry nor viral RNA production were sufficient for miR-7a upregulation, indicating that synthesis of a viral protein is required.

RESULTS

Analysis of mouse miRNAs in Solexa libraries of MCMV-infected cells

To look at MCMV miRNA production and to analyse the impact of MCMV infection on the host miRNA system, we generated small-RNA libraries from NIH 3T3 fibroblasts infected with MCMV at m.o.i. of 1 at 72 h post-infection (p.i.) or from non-infected cells. After removal of adaptor sequences and length selection (17–28 nt), 3.9 million reads for mock-infected and 2.8 million reads for MCMV-infected cells were obtained (Table 1). With no mismatches allowed, 78.6 % of the reads from mock-infected cells and 75.6 % of the reads from MCMV-infected cells could be mapped to either the mouse or the MCMV genome.

The majority of reads from total RNA, from both mock- or MCMV-infected cells, were cellular miRNAs (70.76 and 52.89 %, respectively). rRNAs and tRNAs accounted only for a small fraction of the reads (1.22 % in mock and 2.23 % in MCMV-infected cells). Of the annotated mouse miRNAs, ~30 % could be detected in NIH 3T3 cells with at least ten reads (see Table S1, available in JGV Online). Whilst most miRNAs showed similar read numbers for both mock- and MCMV-infected cells, some outliers were noted (Fig. 1a). miRNAs with a more than tenfold difference and represented by at least 1000 reads were let-7g, miR-7a, miR-26a, miR-27a, miR-125b-3p and miR-199a-5p. We used qPCR to verify these results. Total RNA was isolated from mock- or MCMV-infected cells at 72 h p.i. and qPCR was performed for the 56 most abundant miRNAs (Table S2). Whilst most miRNAs were expressed at a slightly lower level in MCMV-infected cells (possibly an artefact of normalization), three miRNAs showed a greater than fourfold difference (Fig. 1b). As already demonstrated by Buck et al. (2010), miR-27a was drastically reduced in MCMV-infected cells, as was miR-26a, although to a lesser degree. Interestingly, one mouse miRNA, miR-7a, was highly upregulated (Table 2).

Analysis of MCMV miRNAs in deep-sequencing libraries of MCMV-infected cells

Only 7.83 % of the total reads in MCMV-infected cells, accounting for 13 % of all miRNA reads, mapped to viral miRNAs (Table 1). This is in contrast to previous observations (Dölkken et al., 2007) where a small-RNA cloning approach showed the majority of miRNAs to be of viral origin at 72 h p.i. This discrepancy was not due to differences in the m.o.i., as the same protocol was used (m.o.i. = 1 plus centrifugal enhancement). Furthermore, a higher m.o.i. (2.5 and 5) did not lead to more abundant viral miRNAs, as determined by Northern blotting (Fig. S1).

<table>
<thead>
<tr>
<th></th>
<th>Mock-infected cells</th>
<th>MCMV-infected cells</th>
<th>Ago1 IP</th>
<th>Ago2 IP</th>
<th>Fraction of miRNAs</th>
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<tbody>
<tr>
<td>Viral miRNA</td>
<td>0.00 %</td>
<td>7.83 %</td>
<td>1.35 %</td>
<td>0.80 %</td>
<td>12.82 %</td>
</tr>
<tr>
<td>Viral pre-miRNAs</td>
<td>0.00 %</td>
<td>9.86 %</td>
<td>1.62 %</td>
<td>1.28 %</td>
<td>–</td>
</tr>
<tr>
<td>Mouse miRNA</td>
<td>70.76 %</td>
<td>52.89 %</td>
<td>21.53 %</td>
<td>36.93 %</td>
<td>87.18 %</td>
</tr>
<tr>
<td>Mouse pre-miRNAs</td>
<td>76.13 %</td>
<td>58.68 %</td>
<td>24.62 %</td>
<td>40.95 %</td>
<td>–</td>
</tr>
<tr>
<td>Mouse rRNAs</td>
<td>0.64 %</td>
<td>1.51 %</td>
<td>1.85 %</td>
<td>1.10 %</td>
<td>–</td>
</tr>
<tr>
<td>Mouse rRNAs</td>
<td>0.58 %</td>
<td>0.72 %</td>
<td>47.97 %</td>
<td>32.73 %</td>
<td>–</td>
</tr>
<tr>
<td>Mouse and MCMV genome</td>
<td>77.05 %</td>
<td>71.91 %</td>
<td>74.72 %</td>
<td>75.85 %</td>
<td>–</td>
</tr>
<tr>
<td>Total number of reads</td>
<td>3 908 549</td>
<td>2 850 603</td>
<td>4 741 416</td>
<td>3 675 192</td>
<td>1 731 426</td>
</tr>
</tbody>
</table>
All previously annotated MCMV miRNAs (Dölken et al., 2007) could be accounted for, with the exception of m88-1*, which was not found in any library (Table S3). Whilst some reads also mapped to non-hairpin regions of the MCMV genome (0.67% of reads), these reads were spread over the entire genome at low numbers and did not show any additional clusters that could be folded into stem-loop structures (data not shown).

For the m01-1 stem-loop, a new miRNA was found, designated m01-1-3p (Fig. 2a; structure calculated using Mfold, Zuker, 2003), following previous conventions (Dölken et al., 2007; Pfeffer et al., 2005). It was detected in almost equal numbers to the miRNA m01-1 on the opposite strand (1511 vs 1758 reads), which in this study is referred to as m01-1-5p. This miRNA was also observed in a recent study of miRNAs associated with tagged Ago2 following MCMV infection (Libri et al., 2012). Its expression could be confirmed by qPCR by 24 h p.i. at slightly lower levels than that of m01-1-5p (Fig. 2b) and showed similar levels at both 48 and 72 h p.i., whereas m01-1-5p increased in expression up to 72 h p.i. Expression of both m01-1-3p and m01-1-5p could also be confirmed by Northern blotting (Fig. 2c).

Analysis of Solexa libraries after RISC IP

To check for sorting of viral miRNAs into RISCs, we used RNA isolated after RISC IP with antibodies specific for either Ago1 or Ago2 to generate small-RNA libraries. To show that the antibodies used were specific, GFP-tagged Ago1 and Ago2 were precipitated with anti-GFP beads and detected by Western blotting with the antibodies against Ago1 and Ago2 that were used for the RISC IP. No cross-reaction in Western blot detection between Ago1 and Ago2 was observed. For the commercially available Ago1 antibody, the reverse reaction proved to be efficient and specific (Fig. 3a). The Ago2 antibody has been characterized previously by Zhu et al. (2010). The suitability of both antibodies for immunoprecipitation was also verified by qPCR. Mouse miRNAs were measured after IP using antibodies against Ago1, Ago2 or myc and compared with input values (Fig. 3b). Whilst the Ago1 antibody was more efficient than the Ago2 antibody, both clearly produced a higher recovery level than the control anti-myc antibody.

After sequencing of the Ago-associated small RNAs, 4.7 million reads and 3.6 million reads for the Ago1 and Ago2 libraries, respectively, were mapped against both mouse and MCMV sequences (Table 1). In contrast to what was observed for total RNA, a large number of reads mapped to mouse rRNAs, indicating that the RISC IP co-precipitated ribosomes presumably associated with target miRNAs. All detected miRNAs of viral or endogenous origin were loaded in both Ago1- and Ago2-containing RISCs to a similar extent (linear regression of Ago1 versus Ago2 loading: $R^2=0.88$ for mouse miRNAs, $R^2=0.89$ for MCMV miRNAs, Fig. 3c and Table S3). Viral miRNAs accounted for only 6 and 2% (Ago1 IP and Ago2 IP, respectively) of all loaded miRNAs as opposed to the 13% in total RNA. The qPCR data after IP with either Ago1 or Ago2 antibodies also showed essentially equal values for Ago1 and Ago2 loading (Fig. 3d) and was performed for the same mouse miRNAs as in Fig. 1(b), as well as all MCMV miRNAs (Table S2). A comparison of the qPCR results between input and IP did not confirm the reduced incorporation rate of MCMV miRNAs, possibly due to experimental noise, which may well have been within the range of the observed difference. Conservatively, we concluded that viral miRNAs are not preferentially incorporated into RISC.

Kinetics of miR-7a and miR-26a expression changes

To analyse the expression of miR-7a and miR-26a over the time course of infection in fibroblasts as well as in a different cell line, RNA was isolated from NIH 3T3 fibroblasts and

**Table 2.** Fold differences compared with mock infection for miR-7a, miR-27a and miR-26a

<table>
<thead>
<tr>
<th></th>
<th>Solexa reads for total RNA</th>
<th>qPCR data for total RNA</th>
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<tbody>
<tr>
<td>miR-7a</td>
<td>12.95</td>
<td>12.67</td>
</tr>
<tr>
<td>miR-27a</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>miR-26a</td>
<td>0.10</td>
<td>0.22</td>
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TCMK-1 epithelial cells at 24, 48 and 72 h p.i. and from mock-infected cells. We then performed Northern blotting to detect miR-7a and miR-26a; miR-27a served as a control for a differentially expressed miRNA and miR-16 was used for normalization as its level does not change following MCMV infection (Fig. 4a).

Quantitative analysis of three independent Northern blots showed that miR-7a was slightly upregulated at 24 h p.i. but was strongly upregulated from 48 h p.i. onwards in both NIH 3T3 and TCMK-1 cells. In contrast, miR-26a displayed a steady downregulation in NIH 3T3 cells but was marginally upregulated in TCMK-1 cells (Fig. 4b). The qPCR results for miRNA expression in TCMK-1 cells confirmed these results (data not shown). Therefore, miR-26a downregulation appeared to be cell-type dependent.

### Requirements of miR-7a and miR-26a regulation

Downregulation of miR-27a is a post-transcriptional event mediated through a viral RNA that contains a single miR-27-binding site (Buck et al., 2010; Libri et al., 2012; Marcinowski et al., 2012). For miR-26a, a similar mechanism is possible, whilst there are presumably different requirements for the upregulation of miR-7a. UV-inactivated MCMV did not induce changes in miRNA levels either by qPCR or by Northern blot analysis at an m.o.i. of 1 at 72 h p.i. (Fig. 5a, b), indicating that virus entry is not sufficient. Treatment with PAA, which specifically blocks the viral DNA polymerase, showed that amplification of the viral genome is necessary for the full extent of upregulation, consistent with a late effect (Fig. 5a, b).

Treatment with cycloheximide (CHX) blocks protein synthesis but allows viral RNAs to be transcribed. Analysis of miRNA changes was performed at an m.o.i. of 10 at 24 h p.i., as later time points could not be analysed due to the toxicity of CHX. As described previously, miR-27a was rapidly downregulated under these conditions, but no change in miR-7a and miR-26a levels occurred (Fig. 5c, d). The upregulation of miR-7a could be the result of de novo transcription or an extended half-life of the miRNA. Mapping of the Solexa libraries to the two miR-7a-1 and miR-7a-2 precursors revealed that only miR-7a-1 was transcribed. The miR-7a-1 hairpin is located in an intron of the hnrnpk gene, whilst miR-7a-2 is intergenic. qPCR analysis of hnrnpk mRNA showed no upregulation during MCMV infection (data not shown), indicating that pri-miR-7a transcription was specifically induced and was not a secondary consequence of a change in expression of the host gene.

### Effects of miRNA mimics or inhibitors on virus replication

The strong upregulation of miR-7a may influence MCMV infection similar to the antiviral effect demonstrated for miR-27a (Buck et al., 2010). To test for the effect of miR-7a inhibition and overexpression on virus replication in cell culture, we transfected NIH 3T3 cells with mimics or inhibitors for miR-7a, miR-26a and miR-27a, as well as with a corresponding unrelated control. Cells were then infected with MCMV and virus yield was determined (Fig. 6a, b). We did not observe significant changes relative to the control for any miRNA inhibitor or mimic. Therefore, the tested miRNAs do not appear to significantly modulate MCMV replication in cell culture as assayed by our approach. To verify that the mimic and inhibitor were functional upon transfection, NIH 3T3 cells were co-transfected with 50 nM mimic miR-7, mimic plus inhibitor miR-7 or a

![Fig. 2. Expression of MCMV miR-m01-1. (a) Schematic of the m01-1 hairpin with the novel m01-1-3p sequence GUCAGACUUAAUCUCUUCUUCUCU. (b) qPCR analysis of total RNA isolated from NIH 3T3 cells infected with MCMV (m.o.i.=1) at 24, 48 and 72 h p.i. (c) Northern blots probed with m01-1-3p, m01-1-5p and U6 small nuclear RNA (snRNA). RNA was isolated from NIH 3T3 cells infected with MCMV (m.o.i.=1) at 24, 48 and 72 h p.i. or mock infected.](https://www.microbiologyresearch.org/journal/article-pdf/93/3/1540/10398484/93-3-1540-dittmer-and-forstemann)
control together with a reporter plasmid containing four perfect miR-7a-binding sites or an unrelated sequence in the 3′ untranslated region (UTR) of GFP. The miR-7a mimic reduced GFP expression by 96% in comparison with the control (Fig. 6c). If both the miR-7a mimic and the miR-7a inhibitor were transfected, GFP expressed was only reduced by 73% (Fig. 6c). Neither the miR-7a mimic nor the mimic plus inhibitor combination had any effect on the control plasmid. Thus, both the miR-7a mimic and the inhibitor had readily observable and specific effects in a miR-7a reporter system. We determined the transfection efficiency to be 55 ± 5% (mean ± sd, n=7) in our experiments with flow cytometry using the control reporter. As the GFP expression plasmid must reach the nucleus whereas the mimic and inhibitor act in the cytoplasm, we assumed that the transfection efficiency was higher for these compounds. This has been observed in Drosophila S2 cells where transfection efficiencies with plasmids using lipid transfection reagents do not exceed 70%, but a stably integrated, clonal miRNA reporter can be de-repressed in essentially all cells following transfection of an inhibitor (Förstemann et al., 2007). Thus, we estimated that a large fraction of the cells was loaded with the miRNA mimic or inhibitor in our experiments.

Fig. 3. Analyses of RISC IP. (a) NIH 3T3 cells were transfected with GFP-Ago1, GFP-Ago2 or GFP alone for 48 h and IP was performed on an equal amount of cell lysate with GFP-Trap beads or Ago1 and Ago2 antibodies. Western blots were performed with antibodies against Ago1, Ago2 or GFP alone. Input was 10% of the immunoprecipitate. (b) qPCR data of mouse miRNAs immunoprecipitated with anti-Ago1, anti-Ago2 or anti-myc versus input. Arrows indicate controls (U6, Met-tRNA). (c, d) Solexa read numbers (log2) (c) and PCR Ct values (d) normalized to controls for both mouse and MCMV miRNAs loaded into Ago1 versus Ago2. Ct values for (b) and (d) are shown in reverse order for easy comparison with (c).
DISCUSSION

In this study, we used a deep-sequencing approach to gain further insight into the small RNAs expressed in mouse fibroblasts during MCMV infection.

Mapping of the reads obtained at 72 h p.i. showed that only ~13% of all miRNA reads were of viral origin (Table 1). This is in contrast to the observations made by Dölken et al. (2007), who demonstrated 61% viral miRNAs late after infection using a small-RNA cloning approach. It has
been suggested that MCMV miRNAs may outcompete and thereby disrupt global cellular miRNA expression, given that Kaposi’s sarcoma-associated herpesvirus miRNAs also are highly overexpressed (Umbach & Cullen, 2010) and adenoviral virus-associated RNAs have been implicated as competitive inhibitors for the miRNA pathway components Exportin-5, Dicer and RISC (Andersson et al., 2005; Lu & Cullen, 2004; Xu et al., 2007). Our numbers concerning MCMV miRNAs both in total RNA and after RISC IP, however, did not confirm this hypothesis. Using a competitive inhibitors for the miRNA pathway components Exportin-5, Dicer and RISC (Andersson et al., 2005; Lu & Cullen, 2004; Xu et al., 2007). Our numbers concerning MCMV miRNAs both in total RNA and after RISC IP, however, did not confirm this hypothesis. Using a higher m.o.i. in Northern blot experiments did not lead to higher expression levels of the viral miRNA m01-1-4, which was the most abundant miRNA in the study by Dölken et al. (2007) (Fig. S1).

We could confirm expression of all 27 mature MCMV miRNAs described by Dölken et al. (2007) except for m88-1* (Table S3). However, there were more than 100 reads of a small fragment that mapped 12 nt upstream and partially overlapping with m88-1* (data not shown). Furthermore, expression of the other three MCMV miRNAs that had only been described by one single clone could easily be verified. We therefore cannot confirm m88-1* as a MCMV miRNA based on our data. We found one additional miRNA on the m01-1 hairpin, whose presence could be confirmed by qPCR and Northern blotting (Fig. 2). The newly designated m01-1-3p was expressed at similar levels to m01-1-5p. This miRNA was also described in a recent deep-sequencing study (Libri et al., 2012) but was not verified by alternative detection methods. In addition, a small number of reads were distributed along the entire MCMV genome, but there were no further clusters that could be folded into hairpin structures and the majority of these reads were only 18–20 nt.

Read numbers for the miRNAs varied over three orders of magnitude (Table S3), which is in the same range as the numbers from the previous small-RNA cloning approach (Dölken et al., 2007). However, abundance based on the available numbers from both datasets varied considerably. The most abundant MCMV miRNA in our deep-sequencing library, m108-1, which represented ~30 % of all viral miRNA reads, was detected at a lower frequency with the classical cloning strategy, whereas the opposite was true for M23-2. In addition, m01-2* came up with fivefold more reads than the proper m01-2 miRNA. Recently, Raabe et al. (2011) investigated the small-RNA transcriptome of the human pathogen Vibrio cholerae and noted considerable differences depending on the technique employed. They identified several steps in library preparation that might introduce bias and advocated caution in comparisons of absolute numbers between alternatively processed RNA samples. Alon et al. (2011) also reported that multiplexing strategies with bar codes introduced through adaptor ligation, as used in our protocol, can confer significant bias in miRNA expression profiles. As small-RNA cloning and sequencing by traditional means is equally prone to these biases but, in addition, has limitations due to the low sequence coverage, differences in the abundance rank of single miRNAs can be expected and some miRNAs such as m01-1-3p may be missed entirely. However, this does not explain the large gap between the cumulative abundance of viral miRNAs in both datasets (64 vs 13 %). Our qPCR data confirmed the notion that MCMV miRNAs do not constitute the majority of the small-RNA population at 72 h p.i. (e.g. see Fig. 3d). Furthermore, increasing the m.o.i. did not result in a substantially increased production of viral miRNAs (Fig. S2). HCMV miRNAs as measured by

**Fig. 6.** Cellular miRNAs do not appreciably affect MCMV replication in cell culture. (a, b) Yield assay for NIH 3T3 cells transfected with 50 nM inhibitor (I) (a) or mimic (M) (b) for miR-7a, miR-26a, miR-27a, a control miRNA (nc) or blank. Cells were infected at 24 h after transfection at an m.o.i. of 1 with GFP–MCMV, and supernatant was collected over a period of 4 days and used for re-infection of fresh NIH 3T3 cells. The proportion of cells infected in this second round was quantified by flow cytometry (GFP expression) of 10 000 cells. (c) Control of miR-7 mimic and inhibitor efficiency. NIH 3T3 cells were co-transfected with 50 nM mimic, mimic plus inhibitor or control miRNA together with the reporter pEGFP-miR-7 containing four miR-7a-binding sites in the 3′ UTR of GFP or pEGFP control. GFP-positive cells were quantified by flow cytometry and set relative to scramble miRNA.
a deep-sequencing approach accounted for 30% of all miRNAs at an m.o.i. of 3 (Stark et al., 2012). Even in this case, the viral miRNAs did not appear to overwhelm the host miRNA system.

The effectors of miRNA-mediated repression are the RISCs, in which the Ago proteins (Pfeffer et al., 2005) associate with the small RNAs. Mammalian cells encode four Ago-like subfamily proteins (Ago1–Ago4) with mostly redundant functions that also associate equally with miRNAs, even though only Ago2 can cleave target miRNAs (Azuma-Mukai et al., 2008; Liu et al., 2004; Meister et al., 2004; Su et al., 2009). In contrast to Dro sophila, where small RNAs are actively sorted based on differences in structure (Czech et al., 2009; Förstemann et al., 2007; Ghildiyal et al., 2010; Okamura et al., 2009; Tomari et al., 2007), there seems to be no specific sorting mechanism in mammalian cells. However, it has also been reported that Ago1 and Ago2 but not Ago3 and Ago4 possess strand-dissociating activity of miRNA duplexes and function as RNA chaperones (Wang et al., 2009a) and that, at least for endogenous small hairpin RNAs, thermodynamic stability greatly influences the loading process (Gu et al., 2011).

To check to what extent viral miRNAs are loaded into RISCs, we performed RISC IP with non-cross-reactive antibodies against Ago1 or Ago2 (Fig. 3a) and then generated small-RNA libraries as well as qPCR data. All MCMV miRNAs could be in found in both Ago1- and Ago2-containing RISCs (Table S3). Whilst Solexa reads showed some differences (Fig. 3b), qPCR values correlated well between Ago1 and Ago2 (Fig. 3c), showing that MCMV miRNAs are also similarly loaded into both Agos.

For the host miRNAs, we could show that Solexa read numbers correlated well between mock- and MCMV-infected cells, although a few miRNAs showed a greater than tenfold discrepancy (Fig. 1a). The same is also true for both HCMV lytic and latent infections, where only a few cellular miRNAs are altered substantially (Poole et al., 2011; Stark et al., 2012).

qPCR and analysis of Northern blots confirmed the regulation of miR-7a and miR-26a in NIH 3T3 fibroblasts, but whilst miR-7a was strongly upregulated in epithelial TCMK-1 cells, miR-26a was marginally upregulated rather than downregulated (Fig. 4b). MCMV can infect many different cell types in vivo, and results by Buck et al. (2010) indicated that miR-27a regulation is similarly pervasive. However, our findings with miR-26 indicated that cell type-specific effects can also occur.

Experiments with UV-inactivated virus, PAA and CHX (Fig. 5) showed that miR-7a upregulation depends on virus replication and protein synthesis. Many examples of changes in cellular miRNA expression caused by virus infection are known, and both induction and repression of miRNAs can be due directly to viral factors or indirectly to an activated host-cell response. For example, papillomavirus E6 destabilizes the tumour suppressor p53, a known miR-34a transactivator (Wang et al., 2009b), whilst Epstein–Barr virus LMP1 activates the miR-146a promoter, leading to modulation of lymphocyte signalling pathways (Cameron et al., 2008). In some cases, however, the cause of miRNA regulation remains unclear, such as the suppression of miR-17-5p and miR-20a by human immunodeficiency virus type 1 (HIV-1) infection (Triboulet et al., 2007), which targets a potential co-factor of the HIV-1 Tat transactivator, and of miR-100 and miR-101 by HCMV (Wang et al., 2008), which are part of the TOR signalling pathway. The viral protein responsible for miR-7a upregulation needs to be determined in further experiments, although the requirement for virus replication to amplify the effect of miR-7a argues for a process driven by high levels of viral protein rather than triggering of a cellular gene expression switch.

So far, there are no confirmed targets for mouse miR-7a. Human miR-7, which is identical to mouse miR-7a, has been widely implicated as a tumour suppressor in lung cancer, glioblastoma and breast cancer cells, mostly through down-regulation of the epidermal growth factor receptor (EGFR) (Kefas et al., 2008; Lee et al., 2011; McNees et al., 2012; Xiong et al., 2011). Furthermore, a recent report by Melnick et al. (2011) showed that inhibition of EGFR pathway signalling attenuates MCMV-induced pathogenesis in organ culture. However, EGFR expression in NIH 3T3 cells transfected with a miR-7a mimic or infected with MCMV showed no detectable difference in Western blot analysis (Fig. S2). Human miR-7 has been shown to regulate several critical signalling pathways, for example by targeting focal adhesion kinase, which is essential for the Ras-ERK 1/2 signalling pathway (Wu et al., 2011), and by targeting PIK3CD, mTOR and p70S6K to regulate the PI3K/Akt pathway (Fang et al., 2012). Interestingly, whilst upregulation of miR-7 was not described following HCMV infection (Stark et al., 2012; Wang et al., 2008), other cellular miRNAs that interfere with the mTor pathway were affected (Wang et al., 2008).

We could not demonstrate an effect of the miR-7a mimic or inhibitor on MCMV replication efficiency in cell culture, despite the fact that we could demonstrate their functionality in a reporter system (Fig. 6). The potential significance of miR-7 induction may therefore be related to virus–host interactions beyond the replication and assembly steps that are accessible in cell culture. We also could not reproduce the moderate antiviral effect of miR-27a that was reported previously (Buck et al., 2010). However, this is in line with a screen of large deletion mutants of MCMV, where viruses missing the region containing the m169 transcript responsible for miR-27 degradation showed no attenuation in NIH 3T3 fibroblasts (Marcinowski et al., 2012; Mohr et al., 2008).

Technical differences (yield assay vs plaque assay with different cell lines used for the titre measurements and differences in transfection and infection protocols, as well as different m.o.i. between our study and the experiments by Buck et al., 2010) may also be responsible for this discrepancy. Our yield assay could resolve a twofold
difference in viral titre within technical replicates (data not shown), but we certainly cannot exclude the possibility that more subtle effects of miR-7a or miR-27a inhibitors/mimics have remained undetected in our assay.

As miR-7a is induced following virus infection, cell lines derived from miR-7a knockout mouse embryos should be more informative but are currently unavailable. One of the future challenges will be to study the importance of cellular miRNAs for controlling virus infections in vivo. Viral mutants with an impaired ability to induce miR-7a expression may be a tool for this purpose, but this will probably also blunt other virus-induced changes in gene expression. A more specific tool could be an engineered virus that expresses a miR-7a inhibitor (a so-called miRNA ‘sponge’) (Ebert et al., 2007).

**METHODS**

**Cell lines and viruses.** NIH 3T3 and TCMK-1 cells were grown in Dulbecco’s minimal essential medium supplemented with 10% FBS, 100 U penicillin ml−1 and 0.1 mg streptomycin ml−1. Wild-type MCMV (Smith strain) with an inserted GFP gene under the control of a CMV promoter was generated as described by Wagner et al. (1999). Virus stocks were prepared in M2-10B4 cells as described by Dölken et al. (2007) and titres were determined by counting GFP-positive cells. Cells were infected at a calculated m.o.i. of 1 (CHX assays m.o.i. 0.1) with centrifugal enhancement (30 min, 800 g) and centrifuged (10 min, 5,000 g) to obtain virus pellets. Supernatant from the infected cells was collected over a period of 4 days p.i., and viral yield was determined by re-infection of NIH 3T3 cells and quantification of GFP expression by flow cytometry (BD FACScan flow cytometer; Becton Dickinson). For controls of mimic and inhibitor effects, oligonucleotides comprising four repeats of a perfect miR-7a-binding site or four repeats of an unrelated sequence were used.

**RNA preparation and Northern blotting.** RNA was extracted using Trizol reagent (Invitrogen) and Northern blotting was performed on 20 μg total RNA, as described previously (Forstemann et al., 2005). DNA probes were 5’-labelled with [γ-32P]ATP and polynucleotide kinase (Fermentas). Blots were analysed and quantified by phosphor-imaging using a Typhoon 9400 Imager (Amersham Biosciences) and Multi Gauge v3.0 (Fujifilm).

**Immunoprecipitation and immunoblotting.** Cell lysis, immunoprecipitation and Western blotting were performed essentially as described by Hartig & Forstemann (2011). For RISC IP, 50 μl Protein G Plus/Protein A agarose (Calbiochem) was incubated overnight with 2.5 μl anti-Ago1 mAb (clone 2A7; Wako Chemicals) or 50 μl anti-Ago2 mAb (clone 6F4; Zhu et al., 2010). As a negative control, an anti-c-myc antibody was used (clone 9E10; Santa Cruz Biotechnology). Beads were washed three times with 500 μl lysis buffer [30 mM HEPES (pH 7.4), 100 mM KCl, 2 mM MgAc, 1 mM DTT] containing 1% (v/v) Triton X-100 (Sigma), incubated with 1 mg cell extract for 1 h at 4 °C and washed again. For Western blotting, Ago1 and Ago2 were cloned into pEGFP-N1 (Clontech) and transfected into NIH 3T3 cells using Fugene HD Transfection Reagent (Roche). GFP fusion constructs were precipitated using GFP-Trap_A beads (Chromotek) or anti-Ago1 and anti-Ago2 antibody as described above. Primary antibodies were diluted 1:1000 for anti-Ago1, 1:10 for anti-Ago2 and 1:500 for anti-GFP antibody (Santa Cruz Biotechnology). For detection of EGFR in transfected or infected cells EGFR rabbit mAb (clone C74B9; Cell Signalling) was used at a 1:1,000 dilution.

**Generation of small-RNA libraries.** Small RNAs were isolated, and libraries for Solexa sequencing were prepared essentially as described by Czech et al. (2008) (a detailed protocol is available on request). Solexa sequencing was carried out at Fasteris (Plan-Les-Ouates, Switzerland) and the MPI for Molecular Genetics (Berlin). Output files were mapped onto the target sequences using bowtie (Langmead et al., 2009) with the option −n0 to force selection of perfectly matching sequences only. Pre-processing of sequences and analysis of the bowtie output files were carried out using perl scripts (available on request). tRNA, rRNA and MCMV genome sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank), and miRNA sequences were extracted from miRBase (http://www.mirbase.org/).

**miRNA quantification by qPCR.** RNA from cell lysates (1 μg per reaction) and immunoprecipitates (entire precipitate) was reverse transcribed using a miScript Reverse Transcription kit (Qiagen). The obtained cDNA (1 μl of a 1:5 dilution) was then PCR amplified using a miScript SYBR Green PCR kit (Qiagen) in a 10 μl reaction with the mature miRNA sequence as forward primer (Table S2). PCRs were subjected to 15 min of 94 °C hot-start enzyme activation, and 40 cycles of 94 °C denaturation for 20 s, 55 °C annealing for 30 s and 70 °C elongation for 30 s on an ABI Prism 7000 SDS. C values were normalized either to U6 snRNA as an endogenous reference or to the mean value of five different controls (glyceraldehyde 3-phosphate dehydrogenase (GAPDH), U6 snRNA, 5.8S rRNA, Met-tRNA and Val-tRNA) as indicated in figure legends. Values were compared directly or fold change was calculated using the 2−ΔΔCt method (Livak & Schmittgen, 2001).

**Viral yield assay and miRNA transfections.** NIH 3T3 cells were seeded at 5 × 104 cells per well and transfected with miRNA mimics or inhibitors (50 nM) using Fugene HD Transfection Reagent. Cells were incubated 24 h prior to infection with GFP–MCMV. Supernatant from the infected cells was collected over a period of 4 days p.i., and viral yield was determined by re-infection of NIH 3T3 cells and quantification of GFP expression by flow cytometry (BD FACScan flow cytometer; Becton Dickinson). For controls of mimic and inhibitor effects, oligonucleotides comprising four repeats of a perfect miR-7a-binding site or four repeats of an unrelated sequence were cloned into pEGFP-N1 using the NotI site in the GFP 3′ UTR yielding pEGFP-mir-7-reporter and pEGFP-control-reporter.

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**REFERENCES**


