Inhibition of human cytomegalovirus replication by small interfering RNAs

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Small interfering RNAs (siRNAs) are the mediators of a sequence-specific process of gene silencing called RNA interference (RNAi). Here, we show that synthetic siRNAs against essential gene products of human cytomegalovirus (HCMV) can trigger RNAi in serum-starved, infected primary fibroblasts, as well as in U373 cells, leading to effective inhibition of viral DNA replication. This opens new possibilities for antiviral strategies and for the analysis of viral and cellular genes important for HCMV physiology.

RNA interference (RNAi) is a natural mechanism of post-transcriptional gene silencing that is widely conserved in the world of multicellular organisms and is thought to have evolved as a defence strategy against viruses and transposable genetic elements (Hannon, 2002). The molecular mediators of RNAi are double-stranded RNAs (dsRNAs) of 21–23 nucleotides in length that induce the sequence-specific degradation of homologous RNAs (Fire et al., 1998; Hamilton & Baulcombe, 1999). These short interfering RNAs (siRNAs) have rapidly developed into a powerful experimental tool for manipulating gene expression (McManus & Sharp, 2002). Since exogenous siRNA expression allows the targeted knock-down of virtually any gene, even in mammalian systems (Caplen et al., 2001; Elbashir et al., 2001), siRNA methodology has become broadly applied to many areas, not just for the study of gene functions. At least at an experimental level, siRNA approaches have been shown to be effective against a variety of viruses, amongst them prominent viruses such as human immunodeficiency virus 1 (Capodici et al., 2002; Coburn & Cullen, 2002; Jacque et al., 2002; Martinez et al., 2002; Surabhi & Gaynor, 2002), hepatitis virus C (Kapadia et al., 2003; Randall et al., 2003; Wilson et al., 2003), influenza virus (Ge et al., 2003) and poliovirus (Gitlin et al., 2002).

To date, no reports have been published describing the use of siRNA technology in human herpesvirus infection. For human cytomegalovirus (HCMV), the prototype of the Betaherpesvirus family and an important pathogen in the immunocompromised host, this may have been due to technical limitations, since cell culture infection systems typically rely on growth-arrested primary fibroblasts, which in turn are difficult to transfect efficiently. We therefore set out to optimize the standard transfection protocol from the Tuschl laboratory (Elbashir et al., 2002) for use in serum-starved primary human foreskin fibroblasts (CRL-2429 from ATCC) employing the transfection reagent Oligofectamine (Invitrogen). Transfections were performed in 12-well plates with cells at 80% confluence. Three µl 20 µM siRNA and 3 µl Oligofectamine were diluted in 50 µl and 12 µl OptiMEM (Invitrogen), respectively. After 5 min at room temperature, both solutions were combined. After 20 min, the cell culture medium was aspirated and replaced by the transfection assay, diluted with 400 µl pre-warmed OptiMEM. At 12 h post-transfection, siRNA-containing medium was removed and cells were washed once with pre-warmed medium.

In order to determine whether RNAi could be used to downregulate HCMV gene expression effectively in primary fibroblasts, we first designed two siRNAs against the viral DNA polymerase, the UL54 gene product, which is essential for virus replication and an established target of antiviral therapy. The selected siRNAs targeted the regions 5'-AACGCAAGGAGAGCUGUUCU-3' [siUL54(1)] and 5'-AUGGUAUCAGCGCGUCUC-3' [siUL54(2)] corresponding to nt 1532–1552 and 1243–1263 of the UL54 coding sequence. To ensure specificity, both sequences were subjected to a BLAST search against human EST libraries. No homology with human polymerases or any other eukaryotic gene was found. A published siRNA targeting the enhanced version of the green fluorescent protein (siEGFP) was chosen as a negative control siRNA (Caplen et al., 2001). In addition, we included an established siRNA against cdk1 gene transcripts as a positive control. This allowed us to monitor the cell-type-specific efficiency of the gene knock-down approach via RNAi. All siRNAs were purchased from Dharmacon. By transfecting the positive control siRNA in primary fibroblasts that had been serum starved for 72 h, we were able to demonstrate that the induction of cdk1 expression after serum restimulation could repeatedly be downregulated by approximately 80–90% indicating a high, albeit not complete, transfection efficiency (data not shown). This enabled us to set up an experimental system that relied on the well-established
protocol of infecting cells in G₀. Therefore, fibroblasts were first synchronized by serum starvation, then siRNA transfected and finally infected with HCMV (AD169) (Fig. 1A). Using pre-synchronized cells in G₀ is advantageous for several reasons: (i) the replicative cycle of HCMV can start immediately in this cell-cycle phase (Salvant et al., 1998); (ii) virus replication performance is highest when cells are infected in the state of quiescence (Noris et al., 2002); and (iii) accumulating viral DNA can easily be distinguished from the peak of unreplicated cellular DNA by flow cytometry (see below).

To assess the effectiveness of the two siRNAs targeted at UL54 we examined UL54 mRNA levels in transfected cells 24 h post-infection (p.i.) by ribonuclease protection analysis (RPA). In each hybridization reaction of the RPA, we included two in vitro-transcribed, radioactively labelled probes, one covering nt 802–1278 of the UL54 coding sequence and the other covering nt 50–188 of the coding sequence for the ribosomal protein L32, whose expression is known to be unaffected by HCMV infection (Wiebusch et al., 2003). Indeed, both UL54-directed siRNAs, but not siEGFP, caused a reduction in UL54 mRNA levels in infected cells, whereas L32 levels were virtually unchanged (Fig. 1B). Moreover, the observed UL54 downregulation seemed specific since expression of immediate-early (IE) proteins was not affected by siRNA transfection, as shown by IE1 immunoblot analysis (Fig. 1C). Whilst the effect of siUL54(2) was only moderate, siUL54(1) transfection repeatedly reduced UL54 mRNA levels by 70–80% relative to control-transfected cells, as determined by scanning with an FLA-3000 phosphoimager (Fuji Film). Given the abovementioned transfection efficiency, the residual UL54 mRNA expression in the siUL54(1)-treated cell population is most likely to result from the poorly or even non-transfected subpopulation of cells. Thus, siRNAs can be used effectively to downregulate gene expression in serum-starved, HCMV-infected primary fibroblasts.

To address the question of whether the extent of siUL54(1)-induced RNA interference is sufficient to inhibit viral DNA replication, we next analysed the DNA content of infected cells. Two characteristics of lytic HCMV infection, namely the inhibition of cellular replication licensing in G₁ (Biswas et al., 2003; Wiebusch et al., 2003) and the extraordinarily high replication rate of the viral genome, allow the convenient assessment of newly synthesized viral DNA by propidium iodide staining and flow cytometry. In the absence of cellular DNA synthesis, the newly replicated

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**Fig. 1.** Downregulation of UL54 expression by targeted siRNAs. (A) Experimental set-up. Subconfluent cultures of human primary foreskin fibroblasts were withdrawn from the cell cycle by growth factor deprivation. After 48 h, cells were transfected with different siRNAs. At 24 h p.i., cells were infected with HCMV (m.o.i. = 10). Cells and supernatants were harvested separately at the indicated time points and processed for further analysis. (B) Total RNA from 24 h p.i. was prepared and analysed for UL54 mRNA expression by ribonuclease protection analysis. An L32-specific probe was included in the same assay as a loading control. Relative expression levels were quantified and given as percentages. (C) Expression levels of the immediate-early protein 1 (IE1) were determined by immunoblot analysis of whole-cell lysates. A non-specific background band occurring in all lanes is marked with an asterisk and indicates equal gel loading.
viral DNA causes the G₁ peak to disappear in favour of a pronounced pseudo-S/G₂ peak, which results from infected cells containing, besides their normal 2ⁿ DNA content, the replicated viral genomes (Bresnahan et al., 1996; Lu & Shenk, 1996). Hence, a left to right shift in addition to a broadening of the main DNA peak is the typical finding when G₀ cells are infected with HCMV. Using standard protocols as described previously (Wiebusch & Hagemeier, 1999) this finding becomes most obvious between 48 and 72 h p.i. when the virus replication machinery reaches maximal activity (Fig. 2A, untreated).

The transfection of control EGFP siRNA only marginally influenced viral DNA synthesis (Fig. 2A, siEGFP). In

![Figure 2](image.png)

**Fig. 2.** Inhibition of virus replication by UL54-directed RNA interference. Quiescent fibroblasts were control transfected (+ siEGFP), transfected with siUL54(1), or left untransfected. Subsequently, cell cultures were infected with HCMV (m.o.i. = 10). Where indicated, 50 μM ganciclovir was added after virus adsorption. (A) At the indicated time points, cells were stained with propidium iodide and analysed for DNA content by flow cytometry. DNA histograms contained no cell doublets and were brought to equal cell numbers. (B) An overlay of the different DNA profiles observed at 72 h p.i. (see main text for further explanation). (C) Supernatants of the infected cultures were collected and virus titres were determined twice in duplicate plaque assays.
contrast, siUL54(1) transfection caused a pronounced inhibition of the left to right shift in the DNA histogram indicating that viral DNA replication was inhibited in the majority of infected cells [Fig. 2A, siUL54(1)]. This effect was comparable with that observed after treatment of infected cells with the potent UL54 inhibitor ganciclovir (50 μM final concentration) (Fig. 2A, ganciclovir). However, in comparison with ganciclovir-treated cells, siUL54(1)-transfected cells presented with a slightly higher proportion of cells containing a greater-than-G1 DNA content indicating a subpopulation of cells with residual viral DNA synthesis. This finding is consistent with a less than total transfection efficiency. Superimposing the DNA histograms obtained after 72 h p.i. more clearly demonstrates the difference in DNA content between ganciclovir- and siUL54-treated cells. The hatched area in Fig. 2(B) most likely reflects the subpopulation of cells inefficiently transfected with siUL54(1). The DNA content of this subpopulation of cells coincided with the pseudo-S/G2 peak of untreated cells, further supporting the notion that the imperfect transfection rate resulted in a subpopulation of cells with residual viral DNA replication. This suggests that the effectiveness of siUL54(1)-mediated RNAi was only limited by transfection efficiency.

To confirm further the antiviral potential of UL54-specific siRNAs, we monitored the increase in viral progeny in the supernatants of infected cell cultures. Compared with untreated cells, no significant reduction in the virus titre was observed in control-transfected cells over the time period of 4 days p.i. In contrast, siUL54(1) transfection resulted in a 12-fold reduction in virus titres after 96 h of infection (Fig. 2C). As expected, ganciclovir treatment abolished virus growth. These data are consistent with the results shown in Fig. 2(A) and (B), since the inefficiently transfected, minor subpopulation of siUL54(1)-treated cells (hatched in Fig. 2B) is expected to support virus replication and release of progeny into the cell culture medium. Therefore, these data demonstrate the effectiveness of siRNAs in HCMV-infected fibroblasts.

The above data further suggest that by improving the efficiency of siRNA transfection the antiviral effect of siRNAs directed against essential viral gene products could be expected to approximate to that of chemical inhibitors (e.g. ganciclovir) of virus replication. To test this prediction directly, we used an independent cell culture model of HCMV infection that employs fully permissive U373 cells, which can be infected with siRNAs to greater than 99% (data not shown). In this set of experiments we also targeted a second essential gene product of HCMV, the 86 kDa IE2 protein. The advantage of this was the availability of antibodies directed against IE2 enabling us to follow protein levels of IE2, rather than mRNA levels, as was the case for pUL54 (see Fig. 2). We used an IE2 siRNA directed against a target sequence within exon 5 of the major IE region. Consequently, levels of the IE1 protein, which shares sequences encoded by exons 2 and 3 with IE2,
should not be affected. However, at the same time, we were able to use IE1 as a perfect internal control for the specificity of the knock-down approach by employing an antibody directed against exactly those N-terminal sequences that IE1 and IE2 have in common. Fig. 3(A) shows that the siRNA directed against exon 5 (siIE2) specifically silenced IE2 expression over the entire infection period but left the IE1 protein untouched. In addition, an unrelated siRNA directed against EGFP (siEGFP) did not affect the expression of IE1 or IE2. These results demonstrate that IE2 can be specifically targeted in HCMV-infected U373 cells and, in line with the rationale presented in Fig. 2, further show that targets (such as IE2) can be completely downregulated in infected cells that have a high transfection efficiency. Consistent with the complete knock-down of IE2 achieved in these cells, we were unable to recover significant amounts of viral progeny from infected U373 cells transfected with siIE2, whereas control-transfected cells, like untreated cells, were not affected (Fig. 3B). This result is in line with several studies demonstrating that IE2 is an essential viral protein (Azad et al., 1993; Heider et al., 2002).

In conclusion, our study provides evidence that siRNA methodology can be successfully used to knock-down selected HCMV genes in various cell culture systems. This implicates the practicality of RNA transfection, even in growth-arrested primary human fibroblasts, although in this case transfection efficiency and/or the selection of the sequence to be targeted have to be carefully optimized. Our results further show that HCMV has no mechanisms to interfere with the RNAi pathway (at least downstream of the siRNA signal). On the one hand, this will enable new experimental approaches in analysing both viral and cellular gene functions in the context of HCMV infection. However, the high specificity and flexibility of the siRNA approach offers a great advantage over conventional antiviral strategies, which are mainly based on chemical inhibitors of the HCMV polymerase such as ganciclovir, foscarnet or cidofovir. Use of these compounds is limited by their dose-dependent toxic side effects and by rapid selection of drug-resistant virus strains. Although selection of resistant point mutants has also been observed following siRNA treatment (Gitlin et al., 2002), this problem can be tackled relatively simply by the systematic evaluation and application of multiple effective siRNA sequences.

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