

Complete Cure of Persistent Virus Infections by Antiviral siRNAs

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Small interfering RNAs (siRNAs) have been developed as antiviral agents for mammalian cells. The capacity of specific siRNAs to prevent virus infections has been demonstrated, and there is evidence that these new antiviral agents could have a partial therapeutic effect a few days after infection. We investigated the possibility of curing a persistent infection, several months after becoming established, using an *in vitro* model of persistent poliovirus (PV) infection in HEp-2 cells. Despite high virus titers and the presence of PV mutants, repeated treatment with a mixture of two siRNAs targeting both noncoding and coding regions, one of them in a highly conserved region, resulted in the complete cure of the majority of persistently infected cultures. No escape mutants emerged in treated cultures. The antiviral effect of specific siRNAs, consistent with a mechanism of RNA interference, correlated with a decrease in the amount of viral RNA, until its complete disappearance, resulting in cultures cured of virions and viral RNA.

Key Words: RNAi, interference, posttranscriptional gene silencing, siRNA, enterovirus, poliovirus, persistent infection, chronic infection, antiviral

INTRODUCTION

Posttranscriptional gene silencing is a natural, conserved mechanism, present in most eukaryotes and conferring antiviral immunity in plants [1]. T. Tuschl and colleagues [2] demonstrated that synthetic 21-nucleotide-long RNA duplexes transfected into cultured cells silenced genes in a sequence-specific manner. These synthetic siRNAs, with 2-nucleotide 3' overhangs, mimic small interfering RNAs (siRNAs) produced by the ribonuclease Dicer by digestion of large double-stranded RNAs. After incorporation into the RNA-induced silencing complex (RISC), one of the siRNA strands acts as guide and selects the homologous target in the mRNA, which is then cleaved and degraded by RNases. One characteristic of siRNAs is that for activity, the guide (or antisense) strand must be perfectly base paired to its target in the corresponding RNA [2]. However, G:U wobble base pairs do not necessarily alter target specificity, probably because they have the same thermodynamic stability as Watson-Crick base pairs, and C:A mismatches are in some cases tolerated [3–5]. Other small RNAs, named micro RNAs (miRNAs), are also dependent on Dicer for processing and target mRNA for cleavage or translational repression [6]; it has been shown that siRNAs can also function as miRNAs [7].

Guidelines for the choice of siRNA sequences have been published [2,8]. The effect of siRNAs transfected into mammalian cells is transient; it generally does not last more than a few days [2], and it has been suggested that the continued presence of mRNA may be needed for persistence of siRNAs [9]. DNA vectors and repeated transfections have been used to sustain silencing activity [10,11]. We chose to use repeated transfections in this study because the transfer of siRNAs may be associated with fewer safety concerns than the use of DNA vectors in future therapeutic strategies.

Plus-strand RNA viruses appear to be attractive targets for siRNAs (reviewed in [12]), as their genome functions as both mRNA and replication template. In particular, poliovirus (PV), an enterovirus, has a positive-strand RNA genome enclosed in an icosahedral capsid composed of the four proteins VP1 to VP4 [13]. The large coding region of PV is flanked by 5' and 3' noncoding (NC) regions comprising signals involved in the initiation of viral replication. The PV genome encodes a polyprotein precursor of capsid and non-structural proteins, including the 3D RNA polymerase (3Dpol). Gitlin *et al.* [14] used RNA interference targeting the capsid protein region and the 3Dpol

region of the PV genome in lytic infections; they showed that the viral yield of a one-step growth cycle was much lower in cells transfected by antiviral siRNAs before infection than in cells transfected by negative control siRNAs. Viral escape as a result of exposure to single antiviral siRNAs has been observed in several studies [10,14,15]. The emergence of escape mutants is facilitated by the heterogeneity of viral RNA genomes (quasispecies). For PV and another picornavirus, human hepatitis A virus, combinations of siRNAs were more effective than single siRNAs [14,16].

For most experiments investigating silencing viruses by siRNAs, a preventive approach was used: siRNAs were transfected before virus infection [14,17]. Recently, however, successful therapies based on treatment with siRNA have been reported [18,19]. Nevertheless, to the best of our knowledge, complete cure of cells has never been obtained in therapeutic assays with chemical siRNAs.

Persistent virus infections are major medical and veterinary problems. Examples of agents causing persistent infections include plus-strand RNA viruses such as human hepatitis C virus (HCV) and foot-and-mouth disease virus (FMDV). In the absence of a suitable model for growing HCV, the best results of long-term experiments with cell lines transduced by HCV replicons demonstrated that expression of HCV RNA is substantially reduced by specific siRNAs [20]. Inhibition of FMDV by specific siRNAs has been successful in lytic infections *in vitro* and in acute infections *in vivo* [21,22], but the effects of these siRNAs during persistent infections have not been reported.

There are several difficulties associated with the development of therapies in *in vitro* models mimicking some aspects of *in vivo* persistent virus infections: the treatment is applied late after the initial infection; the ratio of infectious virus to cells may be high; the presence of virus mutants is a common feature of persistent infections [23]; the ratio of plus- to minus-strand RNA may be close to 1, suggesting that the majority of viral RNA in cells may be in a double-stranded form [24]; and finally the cure has to be complete to avoid reinfection of the entire culture by a few virus particles.

Some picornaviruses establish persistent infections in their natural animal hosts, and others are suspected to be responsible for chronic human diseases [23]. Immunodeficient individuals can be chronically infected by PV Sabin strains [25], and PV mutants isolated from the intestine of patients after several months of chronic infection can persistently infect human enterocyte-like cells *in vitro* [26]. We have developed several models for studying the molecular mechanisms contributing to persistent enterovirus infections [23,27]. Here, we report an investigation of the possibility of curing persistent PV infections by treatment with specific antiviral siRNAs several months after infection. Despite the high titers of virus produced by

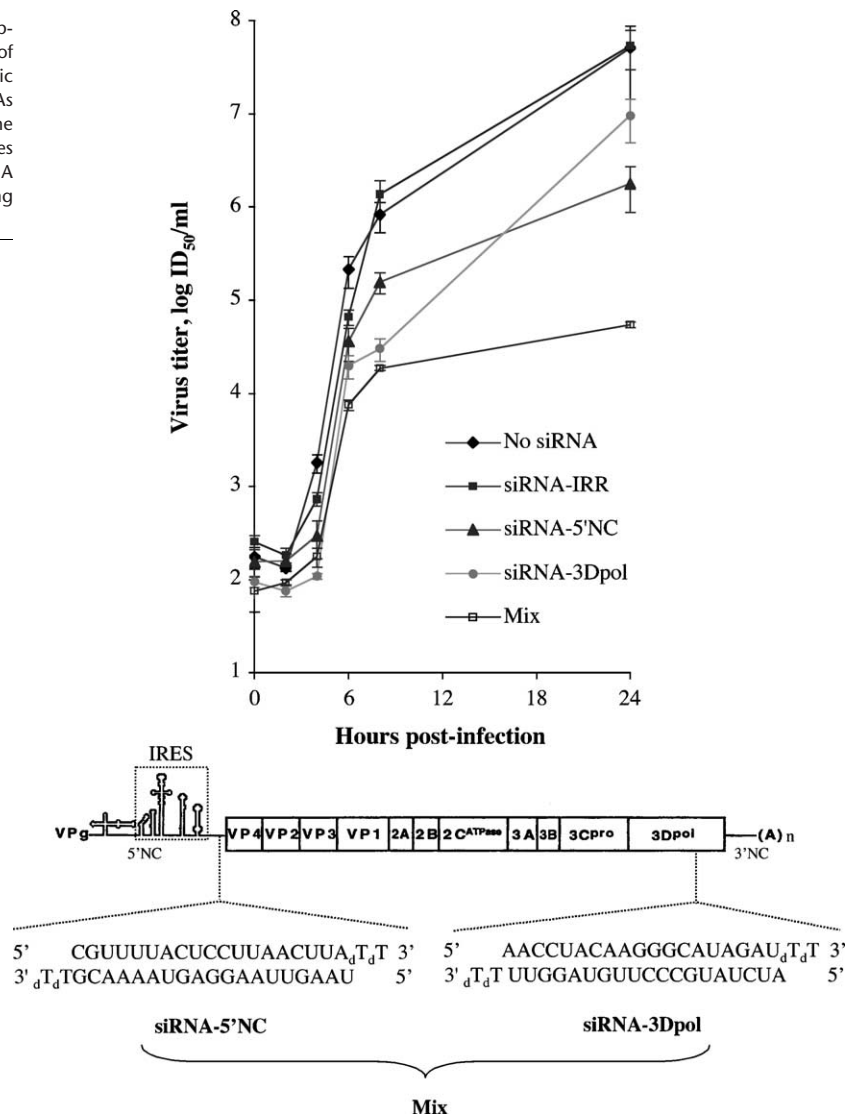
these cells and the presence of PV mutants, repeated treatment with a mixture of two siRNAs induced complete cure of the majority of persistently PV-infected cultures, and our findings are consistent with a mechanism of posttranscriptional silencing.

RESULTS AND DISCUSSION

SiRNAs Directed to the PV 5'NC Region and 3Dpol Gene Reduced the Yield of Lytic and Persistent PV Strains

The reference PV type 3 Sabin strain (Sabin 3) is fully lytic, whereas the L2-2 strain can establish persistent infections in HEp-2 cells at a low multiplicity of infection (m.o.i.) [28]. For effective virus silencing while limiting the risk of silencing undesired cellular genes, we used a combination of only two antiviral siRNAs, targeting sequences separated by about 6200 nt on the viral genome, that meet the criteria defined by Elbashir *et al.* [2] for siRNA targets. One target is in the 5'NC region of the PV type 3 genome between nt 687 and nt 709, outside of the highly structured internal ribosomal entry site (IRES) and upstream of the initiator AUG codon (Fig. 1). The other target is in the 3Dpol region, between nt 6908 and nt 6930, and is conserved among PV serotypes and some other enteroviruses. The corresponding amino acid sequence is part of interface I of 3Dpol oligomers. We assumed that virus displaying mutations in the 3Dpol target would not be viable or would have a reduced growth capacity, and therefore the frequency of emergence of escape mutants would be low. Sabin 3 and L2-2 have identical genomic sequences in both target regions [28]. We transfected HEp-2 cells with siRNAs using Lipofectamine2000 and assessed the growth of the Sabin 3 strain inoculated at an m.o.i. of 10^{-2} infectious doses 50 (ID₅₀) per cell, as described under Materials and Methods. Negative controls included an irrelevant siRNA (siRNA-IRR) and a transfection mixture free of siRNA (Fig. 1). Twenty-four hours postinfection (p.i.), siRNA-5'NC reduced the yield of progeny virus by more than 95%, whereas siRNA-IRR had no significant effect. This result is in agreement with those of Gitlin *et al.* [29], who inserted a let-7 miRNA sequence, acting as an siRNA, at position 703 of the PV type 1 genome. These results suggest that the 5'NC region downstream of the IRES, around nt position 700, can be effectively targeted by siRNAs without significant inhibition by ribosomal scanning during the initiation of translation. The silencing by siRNA-3Dpol was slightly less effective than that by siRNA-5'NC 24 h p.i., possibly because of a secondary structure in the 3Dpol target region. It reduced the yield of progeny virus by more than 80%. However, at earlier times postinfection, siRNA-3Dpol was at least as efficient as siRNA-5'NC (Fig. 1). When used together, the mixture (Mix) of siRNA-5'NC and siRNA-3Dpol reduced the yield by 99.9%, 24 h p.i. These results suggest that at least

FIG. 1. Inhibition of virus growth by antiviral siRNAs. HEp-2 cells were infected with the Sabin 3 strain at a m.o.i. of 10^{-2} ID₅₀, about 20 h after transfection with virus-specific siRNA-5'NC, siRNA-3Dpol, a mixture of both siRNAs (Mix), or the irrelevant siRNA-IRR or with no siRNA. The results shown are means of three to six infected cultures and the SEM are indicated by vertical error scale bars. A diagram of the PV genome is given below, showing antiviral siRNAs targeting the 5'NC and 3Dpol regions.



99.9% of cells were effectively transfected with siRNAs in a single transfection. At a 100-fold higher m.o.i. (1 ID₅₀), the reduction of the yield of Sabin 3 by siRNA-5'NC and siRNA-3Dpol was 94 and 80%, respectively, but the effect of the Mix was similar to that of siRNA-5'NC alone (Fig. 2A). We obtained similar results with the persistent PV strain, L2-2 (Fig. 2A).

To determine whether anti-PV siRNAs can have a therapeutic effect on a persistent PV infection, we infected HEp-2 cells persistently with L2-2 as described previously [28]. We used a persistently L2-2-infected cell line, HEp/L2-2/6, for most of the subsequent experiments: 4 months p.i., the virus titer in the culture medium of HEp/L2-2/6 cells remained high, between 10⁶ and 10⁷ ID₅₀/ml. We performed experiments 4 to 6 months p.i., during which period the genome of the

virus, L2-2m, produced by HEp/L2-2/6, had a mutation (C → U) at nt 698 with respect to the quasispecies consensus sequence. This mutation was in the middle of the target of siRNA-5'NC, whereas the target sequence of siRNA-3Dpol was not mutated. The mutation at position 698 in the genome of L2-2m may correspond to an adaptation of L2-2 to the host HEp-2 cells; it created a wobble base pair between the viral genome and the guide strand of siRNA-5'NC, close to the predicted cleavage site of the RISC. We used L2-2m virus to infect HEp-2 cells pretreated with siRNA-5'NC, and the yield of progeny virus was 75% lower than that of negative controls; the yield from cells pretreated with the Mix was more than 85% lower (Fig. 2A). The silencing effects on L2-2m of the Mix and Mixm (a mixture of siRNA-5'NCm, strictly homologous to the 5'NC target of L2-

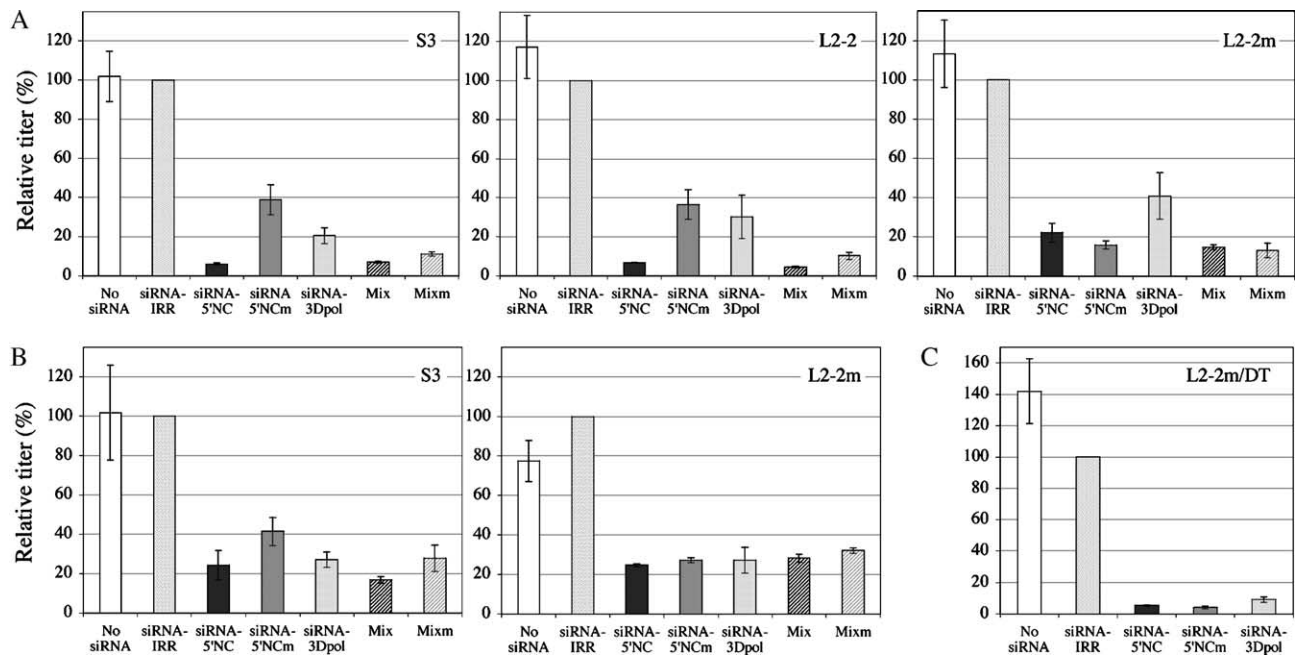


FIG. 2. Yield of progeny virus produced by HEP-2 cells and Vero cells infected at a m.o.i. of 1 ID_{50} , 20 h after transfection by either antiviral or the irrelevant siRNA-IRR, or no siRNA. Samples were harvested 24 h p.i. and the results are expressed as a percentage of the virus yield from cells transfected by siRNA-IRR. Each bar represents the mean value \pm SEM of at least nine or six infected cultures of (A) HEP-2 or (B) Vero cells, respectively. (C) In HEP-2 cells, under the same conditions, the virus L2-2m/DT, produced by a HEP/L2-2/6 culture that was not cured despite two successive transfections with the Mix, was still susceptible to the same specific siRNAs.

2m, and siRNA-3Dpol) were not significantly different. Therefore, the multiplication of L2-2m was silenced by each of anti-PV siRNAs and by their mixtures. Although it was once reported that some mutations in the target sequence resulting in a G:U base pair are sufficient for the virus to escape siRNA inhibition [29], it seems probable that this wobble base pair between PV RNA and the guide strand of siRNA-5'NC, created by the mutation, was recognized as an authentic base pair by the RNA interference machinery, as in other instances [4,5]. Consistent with this possibility, three other siRNAs targeting the same part of the 5'NC region, but corresponding to mutations C \rightarrow A and C \rightarrow G at nt 698 of the target and a 5-nt central mismatch, had little or no silencing activity on L2-2m (Fig. 3). This suggests that silencing of L2-2m RNA by siRNA-5'NC involved RNA degradation rather than inhibition of translation. SiRNA-5'NCm had a moderate silencing effect on Sabin 3 and L2-2 (Fig. 2A), presumably a consequence of the central C:A mismatch between the viral RNA and the guide strand of siRNA-5'NCm. Consistent with this result, the Mixm was slightly less effective than the Mix against the multiplication of these viruses. This result confirms that A:C mismatches are in some cases tolerated [3].

In all of these short-term infections, the negative control siRNA-IRR did not result in a significantly lower

viral yield of Sabin 3, L2-2, or L2-2m than when no siRNA was present (Figs. 1–3). This strongly suggests that the silencing induced by PV-specific siRNAs did not result from activation of Toll-like receptors.

To confirm that the antiviral effects of siRNAs targeting the 5'NC and 3Dpol regions were not the result of modulation of the interferon pathway, we used Vero cells that have a deletion in the type-1 interferon locus [30]. Each of the antiviral siRNAs (siRNA-5'NC, siRNA-5'NCm, and siRNA-3Dpol) inhibited PV strains Sabin 3 and L2-2m similarly in Vero cells and in HEP-2 cells (Figs. 2A and 2B). This strongly suggests that the antiviral effect did not result from a generalized interferon response.

Viral Inhibition by Specific siRNAs in Infected Cells Correlates with a Decrease in the Amount of Viral RNA

We analyzed viral RNA in short-term lytic and long-term persistent infections of HEP-2 cells by Northern blotting to investigate whether the antiviral effects of siRNA-5'NC, siRNA-5'NCm, and siRNA-3Dpol correlated with a decrease in the amount of viral RNA.

For short-term infections, we used two viral strains: L2-2, the strain initially used to establish the persistent infection of HEP/L2-2/6, and L2-2m, the mutated virus produced by this cell line a few months postinfection. We pretreated HEP-2 cells with siRNAs and then

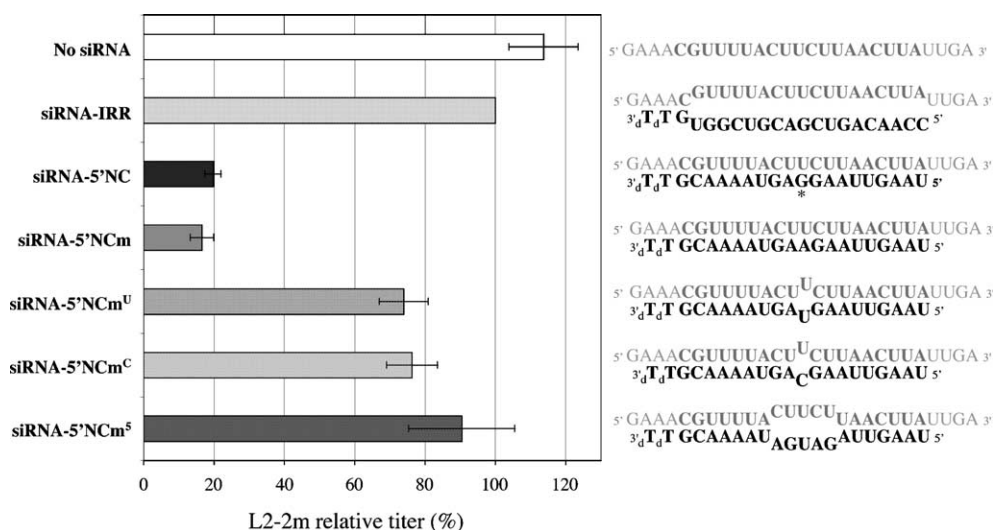


FIG. 3. Effects of central mismatches in siRNAs targeting the 5'NC region of PV on the multiplication of L2-2m in HEp-2 cells. True mismatches between the guide strand of siRNA (black) and the L2-2m genome (gray) are indicated; the wobble base pair is shown with an asterisk. Experimental conditions were the same as those for Fig. 2. The results are expressed as percentages of the virus yield from cells transfected by siRNA-IRR. Each bar represents the mean value of nine infected cultures from three experiments \pm SEM.

infected them with each virus at a m.o.i. of 10 ID₅₀, as described under Materials and Methods. Five hours after infection, as expected, we clearly detected viral RNA with the same electrophoretic mobility as genomic PV RNA in cells treated with the negative control siRNA-IRR (Fig. 4A). In contrast, there was very much less viral RNA in cells treated with siRNA-5'NC, siRNA-5'NCm, siRNA-3Dpol, Mix, or Mixm. Similar results were obtained with the Sabin 3 strain (not shown). In cells transfected with siRNA-5'NC and infected with L2-2m, the amount of viral RNA was one-third of that in cells transfected with siRNA-IRR. This reduction was very similar to those obtained with siRNA-5'NCm and siRNA-3Dpol, which are strictly homologous to the target of the L2-2m genome (Figs. 4A and 4B). These results suggest that siRNA-5'NC, like siRNA-5'NCm, induced L2-2m RNA degradation despite the wobble base pair in the middle of the target. However, inhibition of translation cannot be excluded, because PV replication depends on early translation of the viral genome.

In persistently infected HEp/L2-2/6 cells, 6 months postinfection, PV RNA was detected in cells treated with the negative control siRNA-IRR, as in lytic infections. Twenty-four hours after transfection of HEp/L2-2/6 cells with antiviral siRNA-5'NC, siRNA-5'NCm, siRNA-3Dpol, Mix, and Mixm, the amount of viral RNA fell by 53 to 77% (Figs. 4C and 4D).

Thus, in both lytically and persistently PV-infected cells, antiviral siRNA treatment induced a decrease in the amount of viral RNA, and the results are consistent with a mechanism of RNA interference.

A Mixture of Both siRNAs Directed to the PV 5'NC Region and 3Dpol-Encoding Region Has a Therapeutic Effect on Persistent L2-2 Infections

The preexistence of viral sequence diversity may affect siRNA effectiveness in persistent infections, as there could have been hundreds or thousands of virus generations since the initial infection [31] and the emergence of viral mutants may contribute to the mechanism of persistence (reviewed in [23]). We assayed the therapeutic effects of antiviral siRNAs on HEp/L2-2/6 cells 4 to 6 months p.i. We used the mixtures rather than single siRNAs to prevent the emergence of escape mutants. Negative controls included siRNA-IRR and no siRNA. Any strategy aimed at curing persistent infections must reach the maximum of cells in cultures: it was thus important to transfect all of the cells (5×10^5) in each culture. The relative yield of progeny virus produced by HEp/L2-2/6 cells, 7 and 14 days after a single transfection with either the Mix or the Mixm, was not significantly reduced, whereas the mean values for virus production, 14 days after a double transfection (DT) with either the Mix or the Mixm, were 5 ± 3 and $8 \pm 2\%$, respectively, of that in cultures transfected with siRNA-IRR (see figure in supplementary material). Therefore, we performed a minimum of two transfections.

We performed preliminary experiments with HEp/L2-2/6 cultures to compare the therapeutic effects of the Mix and the Mixm. Two weeks posttransfection, the amount of viral RNA in HEp/L2-2/6 cultures doubly transfected with either the Mix or the Mixm was about 28 and 33%, respectively, of that in cultures doubly transfected with siRNA-IRR (data not shown). Three of the six cultures

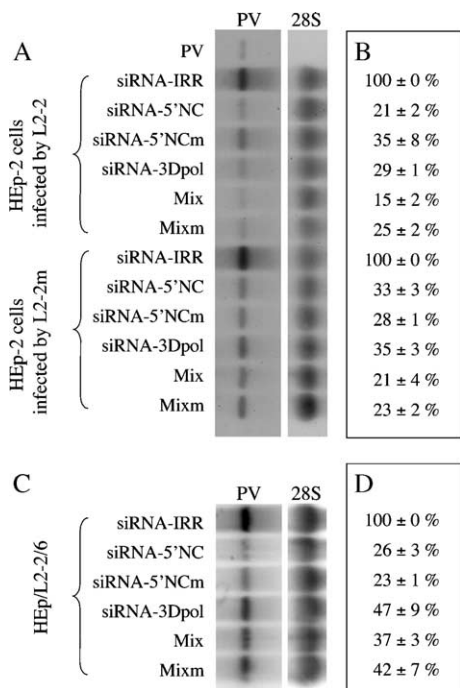


FIG. 4. Detection of viral RNA in cells transfected with either antiviral or an irrelevant siRNA, by Northern blot hybridization. Cells were transfected with either siRNA-IRR or one of the following antiviral siRNAs: siRNA-5'NC, siRNA-5'NCm, siRNA-3Dpol, Mix (siRNA-5'NC and siRNA-3Dpol), or Mixm (siRNA-5'NCm and siRNA-3Dpol). RNAs were extracted from infected cells, separated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a Sabin 3 PV-specific 32 P-labeled probe. The PV probe corresponds to nt 164 to 1186. A second hybridization was performed with a 28S-specific probe to detect this ribosomal RNA (6.3 kb) as a loading control. Probes (PV and 28S) are indicated above the autoradiographs. (A) Short-term infections. HEp-2 cells were infected with the indicated viral strains about 20 h after transfection with siRNAs, at a m.o.i. of 10 ID₅₀, and total RNA was extracted 5 h postinfection as described under Materials and Methods. First lane: a full-length genomic PV transcript was used to indicate the position of virion RNA (7.4 kb). (B) Relative intensities of bands ± SEM, mean values of two experiments. (C) Persistent infection. HEp/L2-2/6 cells were transfected with the indicated siRNAs and total RNA was extracted about 24 h later. (D) Relative intensities of bands ± SEM, mean values of three experiments.

transfected twice with the Mix and two of the six transfected with the Mixm stopped producing virus 5 weeks posttransfection (data not shown). These data suggested that the Mix and Mixm had very similar efficiencies in HEp/L2-2/6 cultures.

We studied the kinetics of virus production following a DT with the Mix. Infectious virus production remained high in the three cultures of HEp/L2-2/6 transfected with siRNA-IRR, whereas it dropped to undetectable levels 34 days after transfection with the Mix in one culture (Fig. 5, top, culture labeled Mix 1).

Although the effect of chemically synthesized siRNAs is usually transient and vanishes after a few days, virus production decreased for about 20 days in all three DT cultures treated with antiviral siRNAs (Fig. 5). This

agrees with the hypothesis proposed by Song *et al.* [9] that the continued presence of target mRNA may be needed for persistence of siRNA. Furthermore, the mechanism of viral persistence in HEp-2 cells relies on an equilibrium between a lytic and an abortive infection [32]; and another, nonexclusive hypothesis is that the prolonged effect of antiviral siRNAs in persistently PV-infected cells may be a consequence of siRNAs modifying this equilibrium.

In two HEp/L2-2/6 cultures treated with the Mix, virus production first declined after transfection, but returned after 3 weeks to the initial level (Fig. 5, top), suggesting

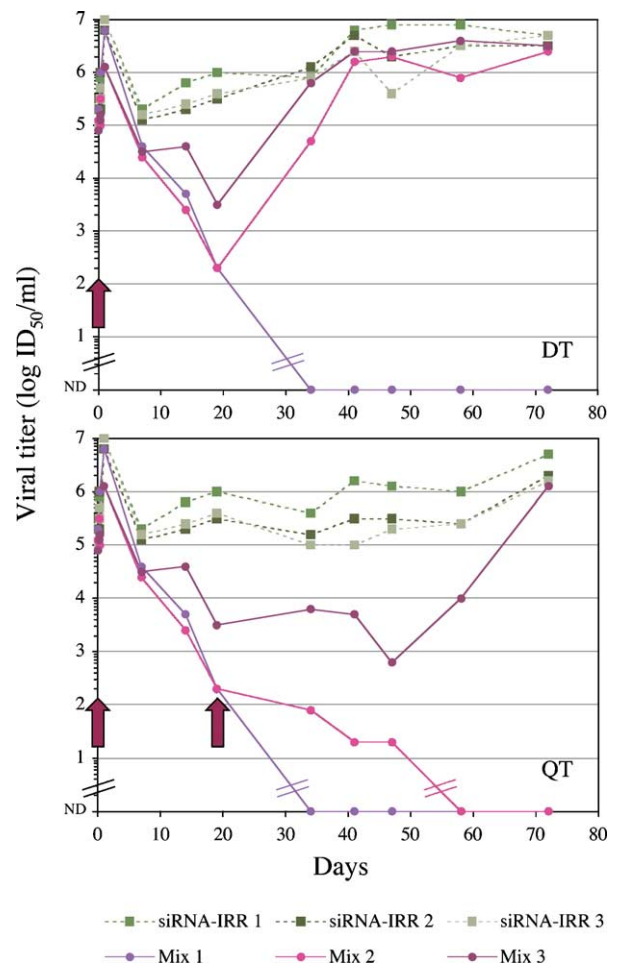


FIG. 5. Decrease in virus production after transfection of persistently PV-infected cultures with a mixture of antiviral siRNAs. HEp/L2-2/6 cells were treated 5 months p.i. Two successive transfections were performed at a 2-h interval with either the Mix (siRNA-5'NC + siRNA-3Dpol) or the irrelevant siRNA-IRR. Cultures were split 20 days later and half of the cultures were again transfected twice with the same siRNAs at a 2-h interval. Cultures transfected twice and four times are denoted DT and QT, respectively. Extracellular virus progeny for each culture was titered at the times indicated after the first double transfection. Double transfections of siRNAs are indicated by arrows. ND, infectious virus not detected. Numbers following the siRNA-IRR and the Mix indicate the culture number.

either that some viral RNA and/or infected cells had escaped antiviral siRNAs or that escape PV mutants had emerged. To prevent this increase in virus production, we performed a second round of DT with the Mix or control siRNA-IRR, 20 days after the first transfections. Cultures transfected four times were denoted QT for quadruple transfection. One culture, not cured by a single set of DT, stopped producing virus after the second set of DT with the Mix, 58 days after the first one (Fig. 5, bottom, culture labeled Mix 2). This suggested that the percentage of cultures cured would be higher after four than after two transfections with the Mix.

Four successive transfections with either the Mix or the Mixm were also performed: the three siRNA-IRR QT cultures remained productive, whereas for each mixture, all three QT cultures stopped producing virus (data not shown), confirming that the Mix and Mixm were similarly effective in HEp/L2-2/6 cultures.

We performed a total of six experiments similar to that presented in Fig. 5 (Fig. 6): 72% of HEp/L2-2/6 cultures stopped producing virus after four transfections with the Mix of antiviral siRNAs, whereas we observed no cures after mock transfections without siRNAs. Most siRNA-IRR-treated cultures continued producing virus so the role of activation of some Toll-like receptors by siRNAs in the cure of persistently infected cells seems improbable, but cannot be excluded. The percentage of cures was significantly higher for QT cultures transfected by the Mix than for both cultures transfected by siRNA-IRR and mock-transfected cultures.

We tested for the presence of PV RNA in HEp/L2-2/6 cultures, which stopped producing virus after transfection with antiviral siRNAs. We used RT-nested PCR of the 5'NC region of the PV genome to determine whether these cultures were completely cured and free of viral genomes. The sensitivity of the method was 0.1 fg of PV RNA, corresponding to about 25 viral genomes or 1 viral genome

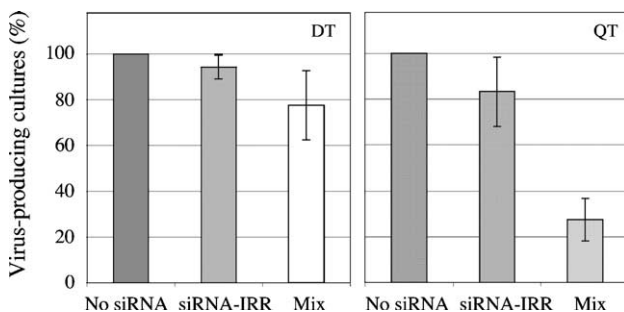


FIG. 6. Therapeutic effects of a mixture of antiviral siRNAs used to transfect persistently PV-infected cultures. A total of six experiments similar to that presented in Fig. 5 were performed and the percentages of virus-producing cultures 1–2 months after the initial transfections with the indicated siRNAs are shown with the SEM (vertical scale bars). The complete cure of cultures transfected by antiviral siRNAs was confirmed by RT-nested PCR, as shown in Fig. 7. DT, double transfection; QT, quadruple transfection.

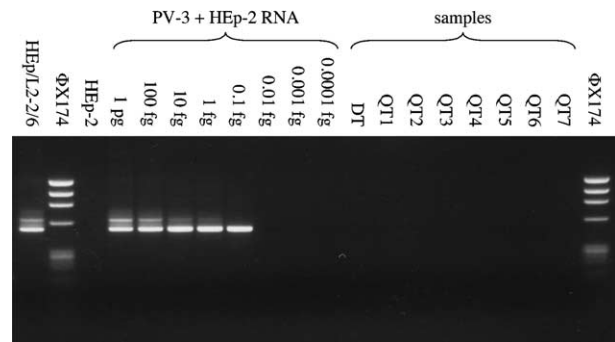


FIG. 7. Assay to detect PV genomes in HEp/L2-2/6 cultures after they stopped producing virus following transfections with antiviral siRNAs. Total RNAs were extracted and viral RNAs were reverse transcribed and amplified by nested PCR in the 5'NC region as described under Materials and Methods. Virus-producing HEp/L2-2/6 and uninfected HEp-2 cells were used as positive and negative controls, respectively. Amplification products corresponding to known amounts of PV type 3 RNA (1 pg to 0.0001 fg) are shown. ΦX174 DNA cleaved by *Hae*III was used as molecular weight markers. DT, double transfection; QT, quadruple transfection.

per 6000 cells. We tested cultures that had stopped producing infectious virus for at least 1 month (4 DT and 13 QT treated with the Mix, 2 DT and 6 QT treated with the Mixm). No viral RNA was detected in these cultures and we therefore considered them to be completely cured (Fig. 7). Thus, after four transfections with the mixtures of antiviral siRNAs, the majority of HEp/L2-2/6 cultures were cleared of both infectious virus particles and viral genomes.

Three of the cured cultures (one DT and two QT treated with the Mix) were tested for their permissivity to PV infection: the progeny yields of Sabin 3 and L2-2m were similar (4×10^8 to 7×10^8 ID₅₀/ml) at 24 h p.i. at an m.o.i. of 1 ID₅₀. When we pretreated the cured cells either with the Mix or the Mixm 24 h before *de novo* infection, we observed a 3-log reduction in viral yield (data not shown). These results indicated that the cured cells were fully permissive to PV infection, like HEp-2 cells; they were also highly susceptible to siRNA transfection, because antiviral siRNAs could efficiently prevent PV multiplication in these cells.

Persistently PV-Infected Cultures That Escaped Antiviral siRNAs Did Not Produce PV Escape Mutants

Even after four transfections with antiviral siRNAs, virus production sometimes rose abruptly about 50 days after the first DT (Fig. 5). To investigate whether PV escape mutants had emerged, we sequenced the genomes of viruses produced by five DT and one QT Mix-treated, but uncured, HEp/L2-2/6 cultures in the regions targeted by siRNAs. No mutation was detected, suggesting that no escape mutations had been selected in the viral genomes at any time during the experiments (75 days), at least in targeted regions. It was possible that mutations had appeared in other regions of the viral genome and modified the secondary structure of the targeted region,

as described for HIV-1 [15]. To test for this, we studied the susceptibility of one virus (L2-2m/DT) from a DT culture and one virus from a QT culture to silencing by siRNA-5'NC, siRNA-5'NCm, and siRNA-3Dpol in HEp-2 cells, 24 h p.i. at a m.o.i. of 1 ID₅₀ (Fig. 2C). Both these viruses remained fully susceptible to silencing by each of the three antiviral siRNAs, confirming that they were not escape mutants. This strongly suggests that, even if mutations appeared in viral genomes outside of targeted regions during siRNA treatment, they did not confer resistance to siRNA. Therefore, the rare failures of antiviral siRNA treatment were probably due to a small number of cells escaping transfection by antiviral siRNAs and repopulating and/or re-infecting the entire culture after several weeks. This agrees with the higher therapeutic efficacy of QT than that of DT. It seems likely therefore that 100% of cultures could be cured by application of a larger number of repeat treatments.

In conclusion, these results indicate that the strategy of repeated transfections with different antiviral siRNAs is sufficient to prevent the emergence of virus escape mutants in persistently infected cultures. They strongly suggest that antiviral siRNAs inhibited viral multiplication in both lytically and persistently infected cells by a mechanism of RNA interference. This is, to our knowledge, the first demonstration of the complete cure by specific antiviral siRNAs of entire cultures of cells productively infected by a human virus. We also show that antiviral siRNAs can completely cure cells persistently infected by virus for several months.

MATERIALS AND METHODS

Cells and viruses. The human epithelial tumor cell line HEp-2 and the monkey kidney cell line Vero (clone VC10 [33]) were propagated at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (NCS). The vaccine Sabin 3 strain (P3/Leon12a1b), lytic in these cell lines, was used as a reference PV strain. The L2-2 PV mutant was isolated and described previously [28]. Virus stocks were prepared in HEp-2 cells at 34 and 37°C for the Sabin 3 and L2-2 PV strains, respectively. Titers were determined in 96-well plates, using an endpoint micromethod [28]. All experiments in cells were carried out at 37°C. To establish persistent infections, HEp-2 cells were infected with L2-2 at a m.o.i. of 10⁻⁴ ID₅₀ and grown at 37°C, in DMEM–2% fetal calf serum for the first week and thereafter in DMEM–10% NCS as described [28]. Persistently L2-2-infected cultures were named HEp/L2-2 followed by the number of the culture. Most experiments were performed with the HEp/L2-2/6 culture, in which no spontaneous cure was ever observed. After 4 months of culture, HEp/L2-2/6 was found to produce a mutant, L2-2m, with a point mutation at nt position 698 (C → U).

siRNAs. The choice of the 21-nt-long sequences was made with the help of the Fuzznuc program (<http://www.bioweb.pasteur.fr/seqanal/interfaces/fuzznuc.html>) according to the recommendations of T. Elbashir and colleagues [2]. Oligoribonucleotides were synthesized either by Genset-Oligos or by C. Gouyette (PT 7, Institut Pasteur). siRNA-5'NC (nt 687–709) and siRNA-3Dpol (nt 6908–6930) are strictly homologous to the corresponding segments of the genome of PV strains Sabin 3 and L2-2. An equimolar mixture of siRNA-5'NC and siRNA-3Dpol was made and named Mix. siRNA-5'NCm differs from siRNA-5'NC by a C → U nucleotide change corresponding to the target sequence of L2-2m. Two

other siRNAs with either an A or a G at the same central position on the sense strand, that is a U or a C on the guide strand, were also produced and named siRNA-5'NCm^U and siRNA-5'NCm^C, respectively (Fig. 3). An additional siRNA, siRNA-5'NCm^S, with a 5-nt central mismatch with the target, was used. A negative irrelevant control, siRNA-IRR (5'-CACCGAC-GUCGACUGUUGG₄T₄T-3'), with the inverted sequence of exon 8 of CD155 (nt 2402–2424) was included in all experiments. To obtain duplex siRNAs, sense and antisense strands at a concentration of 20 mM were annealed, as described [2]. Annealing of oligoribonucleotides was verified by electrophoresis in a 4% NuSieve gel, and the amount of siRNAs was verified with a Fluor-S Multi-Imager (Bio-Rad).

Transfection and infection of cells. siRNA transfections using Lipofectamine 2000 (Invitrogen) and other transfection agents were performed and their efficiencies were compared (see supplementary material). Cells were grown in OptiMEM I Glutamax (abbreviated here to OptiMEM) (Gibco BRL) containing 10% NCS, without antibiotics, for 24 h in 24-well plates, until they reached 90–95% confluence. Cells were then washed and transfected with 15 pmol siRNAs (final concentration of each siRNA 25 nM) mixed with 3 μl of Lipofectamine 2000 in 100 μl of medium per well, as recommended by the manufacturer. After 4 h at 37°C, the serum concentration was adjusted to 10%, and plates were further incubated at 37°C.

For lytic and short-term infections, cells were infected with PV (strain Sabin 3, L2-2, or L2-2m) about 20 h posttransfection, at an m.o.i. of 0.01, 1, or 10 ID₅₀, for 30 min at 37°C. Total virus was recovered after 0, 2, 4, 6, 8, and 24 h at 37°C, clarified, and titered on HEp-2 cells.

Persistently infected HEp/L2-2 cultures were transfected with siRNA as described above, except that they were washed three times before transfection, and 500 μl of OptiMEM was added to each well. One hundred microliters was immediately withdrawn for titration (*t*₀). The siRNA–Lipofectamine mixture (concentration of each siRNA 25 nM) was added to cell monolayers, and cultures were further incubated at 37°C. This procedure is defined as a single transfection. In double transfection assays, the cell supernatant was replaced after 2 h of incubation with 400 μl of OptiMEM and 100 μl of an identical siRNA–Lipofectamine mixture. After 4 h of incubation at 37°C, the serum concentration was adjusted to 10%. Samples of cell supernatant were frozen 4, 6, 8, 10, and 24 h after *t*₀ and then weekly. Some cell cultures were transfected again 20 days after the first DT. The day before transfection, cultures of each well were split 1:2. Half of the cultures were again doubly transfected with siRNAs, as described above. These cultures are denoted QT for quadruple transfection.

Virus and viral RNA detection. Infectious virus in supernatants of HEp/L2-2/6 cells was titered as described [28]. When infectious virus was not detected in any of several undiluted samples of the same culture at various passages, its absence was verified by RT-nested PCR. RNA was extracted from confluent cell cultures and stabilized with the successive use of a RNeasy Protect Mini Kit (Qiagen) and QIAshredder Homogenizer (Qiagen), following the manufacturer's directions. For each cellular extract, the integrity of ribosomal RNAs was verified by electrophoresis in ethidium bromide-stained 1% agarose gel. The sensitivity of the detection method was determined with known amounts of PV type 3 RNA (from 1 pg to 0.0001 fg) transcribed from plasmid pT7L/P1(L2-2) as described [28] and mixed with 1 μg of HEp-2 cellular RNA. The first RT-PCR step was the amplification of the 5'NC region, using primers (nt positions 164–184 and 959–976), RNasin (Promega), and the SuperScript One-Step RT-PCR kit with Platinum *Taq* (Invitrogen). Nested PCR was performed with the same Platinum *Taq* DNA polymerase (Invitrogen) using a sense primer corresponding to nt positions 269–286 and an antisense primer to nt positions 775–795, following the same amplification protocol. PCR products were analyzed by electrophoresis in 1.5% agarose gel.

Viral RNA Extraction and sequencing. cDNAs were prepared from viral RNA, obtained from 2 μl of medium of cultures with a titer ≥10⁶ ID₅₀/ml, in the presence of RNasin (Promega) and 10 pmol of antisense primer (nt positions 1169–1186 and 7441–7464). After 5 min denaturation at 80°C, primer and viral RNA were annealed for 5 min at 50 (for the 5'NC region) or 45°C (for the 3Dpol region) and cDNA was synthesized in 20 μl of a mixture containing 1 mM each dNTP, 2 μl of dithiothreitol (Gibco), and 40 units of Superscript II RNase H⁻ reverse transcriptase (Gibco) in the

enzyme buffer. After incubation for 30 min at 50°C and denaturation for 5 min at 95°C, the cDNA regions between nt 614 and nt 1186 in the 5'NC region and nt 6536 and nt 7464 in the 3Dpol region were amplified [34], in 80 µl with 10 pmol of primers and 2.6 units of Expand High Fidelity PCR System (Roche) according to the manufacturer's instructions. The amplified DNA fragments were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on an ABI Prism DNA 377 sequencer (Applied Biosystems). Nucleotide sequences were aligned with CLUSTAL W [35].

Northern blot analysis of RNA. Transfections with siRNAs and infections were performed as described above. Total RNA was extracted from persistently infected HEp/L2-2/6 cells and from HEp-2 cells 5 h after infection by PV strain Sabin 3, L2-2, or L2-2m, at a m.o.i. of 10 ID₅₀. TRIzol reagent (Life Technologies) was used as recommended by the manufacturer. After denaturation for 10 min at 85°C in 50% formamide, 2.2 M formaldehyde in running buffer as described [36], RNAs from three cell cultures in wells of 24-well plates were run on a 1.2% agarose–0.6 M formaldehyde gel. A full-length genomic PV transcript was used as a marker (7.4 kb). RNAs were transferred to a nylon membrane (Hybond-N; Amersham) and fixed. To prepare the probes, the 5'NC–capsid region between nt 164 and nt 1186 of the PV Sabin 3 strain genomic template was amplified by PCR [34]. The human ribosomal 28S template was from Ambion. For each probe, 125 mCi of [³²P]UTP (Amersham) was used and ³²P-labeled riboprobes were produced with the MAXIScript kit (Ambion) and purified by following the manufacturer's instructions. Blots were successively hybridized with the PV and ribosomal 28S probes, as described [36]. The intensity of radiolabeled bands was quantified with a STORM 820 phosphorimager (Molecular Dynamics) and analyzed using the Image Quant program (Molecular Dynamics).

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2005.07.697.

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