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## Inhibition of measles virus and subacute sclerosing panencephalitis virus by RNA interference

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### Abstract

Subacute sclerosing panencephalitis (SSPE) is a rare, but fatal outcome of measles virus (MeV) infection. SSPE develops after prolonged persistence of mutated MeV called SSPE virus. Although a combination therapy using interferon and inosiplex or ribavirin appears to prolong survival time to some extent, there is currently no effective treatment to completely cure SSPE and a new treatment strategy is greatly needed. In this study, we adopted RNA interference (RNAi) strategy and examined whether small interfering RNAs (siRNAs) can be used to inhibit replication of MeV and SSPE virus. We report here that siRNAs targeted against L mRNA of MeV, either synthetic siRNAs or those generated by pcPUR + U6i-based expression plasmids, effectively and specifically inhibited replication of both MeV and SSPE virus without exhibiting any cytotoxic effect. The L protein of MeV is a major component of RNA-dependent RNA polymerase that is essential for viral RNA replication, and yet it is least abundant among all the MeV proteins expressed. Therefore, mRNA encoding the L protein would be a good target for RNAi strategy. The present results imply the possibility that our siRNAs against MeV L mRNA are among the potential candidates to be used to treat patients with SSPE.

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### 1. Introduction

Measles virus (MeV), a member of the Genus *Morbilivirus*, the Family *Paramyxoviridae*, is nonsegmented, negative-stranded RNA virus. The MeV genome contains six genes, such as N, P/C/V, M, F, H and L (Lamb and Kolakofsky, 2001). The P/C/V gene is transcribed to the overlapping P/C/V mRNAs while the other genes each to a single mRNA. The MeV genes are flanked by the extragenic 3'-terminal leader and the 5'-terminal trailer regions, and are connected by short stretch of conserved sequences. These gene boundary sequences consist of three regions, called the transcription end, intercistronic and transcriptional start sequences, which function as the transcription regulatory sequences. The L mRNA encodes the L protein that contains the catalytic motif common to the RNA-dependent RNA polymerase of negative-strand RNA viruses.

Subacute sclerosing panencephalitis (SSPE) is a rare, but fatal outcome of MeV infection. It has been estimated that approximately 10–20 out of a million MeV-infected individuals develop SSPE, which is caused by mutated MeV, or SSPE virus, after a prolonged incubation period of an average of 7 years (Okuno et al., 1989). Although a combination therapy using interferon (IFN) and inosiplex (Anlar et al., 2004; Gascon, 2003) or ribavirin (Hosoya et al., 2004) appears to improve clinical conditions and survival time to some extent, there is currently no specific treatment available to cure patients with SSPE and a new treatment strategy for SSPE is greatly needed.

RNA interference (RNAi), which involves small interfering RNA (siRNA) of 21–23-bp, is known to play an important role in host defense mechanisms in plants and insects (Bernstein et al., 2001; Elbashir et al., 2001b; Hamilton and Baulcombe, 1999; Zamore et al., 2000). siRNAs function in mammalian cells as well (Elbashir et al., 2001a; Gitlin et al., 2002; McCaffrey et al., 2002; Paddison et al., 2002; Paul et al., 2002; Rubinson et al., 2003; Xia et al., 2002) and are shown to suppress replication of a wide variety of viruses, including human immunodeficiency virus (Coburn and Cullen, 2002; Huelsmann et al., 2006; Jacque

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et al., 2002; Lee et al., 2002; Takaku, 2004), hepatitis B and C viruses (Radhakrishnan et al., 2004; Randall and Rice, 2004), severe acute respiratory syndrome (SARS)-coronavirus (Wang et al., 2004; Wu et al., 2005), human rhinovirus (Phipps et al., 2004), human respiratory syncytial virus and parainfluenza virus (Barik, 2004; Bitko et al., 2005; Zhang et al., 2005), the latter two of which belong to the Family *Paramyxoviridae*. However, little information is available so far on RNAi against MeV and SSPE virus infection. We report here that siRNAs targeted against L mRNA of MeV, either synthetic ones or those generated by pcPUR + U6i-based expression plasmids, effectively inhibit replication of both MeV and SSPE virus. To our knowledge, this is the first report describing effective inhibition of MeV and SSPE virus by means of siRNA.

## 2. Materials and methods

### 2.1. Synthetic siRNAs and plasmids expressing siRNAs

Chemically synthesized RNA oligonucleotides were purchased (Japan Bio Service, Saitama, Japan). In order to increase the stability of the siRNA, 3' overhang of each strand was made of thymidine residues instead of uridine (Elbashir et al., 2001a, 2001b). siRNA duplex was prepared as described previously (Takigawa et al., 2004). In brief, 80  $\mu$ M each of sense and antisense RNA oligonucleotides were heated at 90 °C for 1 min and incubated at 37 °C for 60 min in an annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4), 2 mM magnesium acetate). The siRNA thus prepared was transfected to cultured cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Plasmid vectors expressing siRNAs were constructed as reported previously (Takigawa et al., 2004) with some modifications. Briefly, chemically synthesized DNA oligonucleotides were purchased (Especk Oligo Service Corp., Tsukuba, Ibaraki, Japan). Sense and antisense DNA oligonucleotides were annealed and cloned into the unique *Bsp*MI site of pcPUR + U6i downstream of the U6 promoter (Miyagishi and Taira, 2002). pcPUR + U6i was kindly provided by Dr. K. Taira, Graduate School of Engineering, The University of Tokyo, Tokyo, Japan. The siRNA expression plasmids or an empty control vector were transfected into cultured cells by using FuGene 6 reagent (Roche) according to the manufacturer's instructions.

### 2.2. Cell culture and virus infection

A marmoset B-lymphoblastoid cell line B95a was described previously (Itoh et al., 2002; Katayama et al., 1997; Kobune et al., 1990; Shibahara et al., 1994). Vero/SLAM cells (Ono et al., 2001) were a kind gift from Dr. Y. Yanagi, Department of Virology, Kyusyu University Graduate School of Medicine, Fukuoka, Japan, and were maintained in Dulbecco's modification of Eagle's minimum essential medium supplemented with 10% fetal calf serum and G418 (400  $\mu$ g/ml).

Wild-type MeV strains, clinical isolates K52 and T8, were described previously (Katayama et al., 1997). K52 and T8 strains

belong to MeV genotypes D3 and D5, respectively. A laboratory-adapted standard strain, Edmonston, which belongs to genotype A, was also used as described previously (Shibahara et al., 1994). SSPE-Kobe-1, an SSPE virus strain, was recently isolated from a patient with SSPE, and belongs to genotype D3 (manuscript in preparation). SSPE-Kobe-1 has characteristics similar to other strains of SSPE virus, such as the lack of cell-free virus production and accumulation of mutations in the viral genome (data not shown).

Encephalomyocarditis virus (EMCV; DK-27 strain), a member of the Family *Picornaviridae*, was prepared as described previously (Song et al., 1999; Taguchi et al., 2004) and used as an irrelevant control virus in this study.

### 2.3. Virus titration

Serially diluted culture supernatants of cells infected with wild-type MeV were inoculated onto Vero/SLAM cells and virus adsorption was done at 37 °C for 1 h. After being cultured overnight, the number of syncytium formed on the monolayer cells were counted. To assess the effect of siRNA on SSPE virus replication, SSPE virus-infected cells were transfected with either synthetic siRNA or a pcPUR + U6i expression plasmid, and then cocultured with fresh Vero/SLAM cells. After overnight culture, the number of newly formed syncytia was counted.

EMCV titers were determined by plaque assay on Vero/SLAM cells, as described previously with minor modifications (Song et al., 1999; Taguchi et al., 2004).

## 3. Results

### 3.1. Inhibition of MeV replication by synthetic siRNA

We first assessed the efficacy of synthetic siRNAs, which were designed to target L mRNA of MeV. The sequences and positions of the siRNAs are summarized in Table 1. siRNA targeted against NS3 of hepatitis C virus, named NS3-2 (Takigawa et al., 2004), served as a negative control. In the initial experiments, Vero/SLAM cells were first transfected with siRNA for 5 h and then inoculated with the K52 strain of MeV. After 48 h, virus titers in the culture supernatants were determined. As shown in Fig. 1A, MeV replication was markedly inhibited by siRNAs MV-L2, -L4 and -L5, and moderately by MV-L1, -L3 and -L32. On the other hand, MV-L6 only slightly inhibited MeV replication.

When cells were first inoculated with MeV and then transfected with siRNA, marked inhibition of MeV replication by siRNAs MV-L2, -L4 and -L5 was also observed. Treatment with MV-L2 siRNA after 1, 6 or 12 h postinfection efficiently inhibited MeV replication (Fig. 1B). However, the inhibitory effect was no longer observed when the siRNA was added to the cells 24 h postinfection. Similar results were obtained with MV-L4 and -L5 siRNAs (data not shown). The inhibitory effect was dose-dependent and the 50%-inhibiting dose was 3 nM for MV-L2 (Fig. 1C).

Table 1  
Targeted positions and sequences of siRNA used in this study

siRNA	Position <sup>a</sup>	Sequence
MV-L1	40 to 60 (9273 to 9293)	5'-GUUCACCUAGAUAGCCCGAUA-3'
MV-L2	141 to 161 (9374 to 9394)	5'-GAACAUCAAGCACCGCCUAAA-3'
MV-L3	225 to 245 (9458 to 9478)	5'-GCUUAGGAGUUAUCCGGCCCA-3'
MV-L31	219 to 239 (9452 to 9472)	5'-GUCCAAGCUUAGGAGUUAUCC-3'
MV-L32	273 to 293 (9506 to 9526)	5'-UCAGGAUUUUUUUAACAUAGA-3'
MV-L4	311 to 331 (9544 to 9564)	5'-GGAAGAUCGGUGAGCUCCUAA-3'
MV-L5	429 to 449 (9662 to 9682)	5'-GGACAUCAAGGAGAAAAUUUAU-3'
MV-L6	563 to 583 (9796 to 9816)	5'-GGAGGAGACACACACCUGUAU-3'

<sup>a</sup> Nucleotide positions in the translated region of viral L mRNA. Parentheses indicate the corresponding positions on cDNA to the genomic RNA of the Ichinose-B95a strain of MeV (EMBL/GenBank/DBJ accession number AB016162).

### 3.2. Inhibition of MeV replication by plasmid-mediated siRNA

We constructed pcPUR+U6i-based plasmids expressing MV-L1 to -L5 and MV-L32, and tested their possible inhibitory effects on MeV replication. The NS3-2 siRNA-expressing plasmid served as a negative control. Vero/SLAM cells were transfected with the expression plasmid and then inoculated with the virus. After 2 days, virus replication was monitored. The results clearly demonstrated that pcPUR + U6i-mediated siRNA, MV-L2, -L3, -L4 and -L5, markedly inhibited replication of the K52 strain of MeV (Fig. 2A). The inhibitory effects of MV-L1 and -L32 appeared to be weaker than those of MV-L2 to -L5 in this series of experiments. The plasmid-based siRNA, MV-L2, -L4 and -L5, also inhibited replication of MeV T8 strain, which belongs to genotype D5 and shares the target sequences in common with genotype D3 strains (data not shown). On the other hand, only MV-L2 siRNA, but not MV-L4 or -L5, inhibited replication of the Edmonston strain, which belongs to genotype A (Fig. 2B). It should be noted that there are one and two nucleotide substitutions in the target sequences for MV-L5 and -L4, respectively, between genotypes D3 and A. On the other hand, the target sequence for MV-L2 is completely conserved between the two genotypes. As had been expected, replication of an unrelated virus, EMCV, was not affected by MV-L2 siRNA (Fig. 2C). These results suggested MeV sequence-specific inhibition by the siRNAs.

### 3.3. Inhibition of SSPE virus replication by synthetic siRNA and plasmid-mediated siRNA

We then tested to see whether or not MV-L2 siRNA, either synthetic or pcPUR + U6i-mediated, inhibits SSPE virus infection. Vero/SLAM cells persistently infected with SSPE-Kobe-1 and uninfected cells were transfected with either synthetic siRNA or pcPUR + U6i-MV-L2 overnight. NS3-2 siRNA, either synthetic or plasmid-mediated, served as a control. After transfection, the SSPE virus-infected cells were cocultured with uninfected cells and the number of syncytium that emerged on the monolayer cells was counted 24, 48 and 72 h thereafter. This experimental procedure was used to examine the effect of siRNAs on both virus multiplication in the cell and cell-to-cell viral spread. The results obtained clearly demonstrated that both syn-

thetic and plasmid-mediated MV-L2 siRNA markedly inhibited SSPE virus replication and/or cell-to-cell spread (Fig. 3A and B).

In the next series of experiments with SSPE virus, Vero/SLAM cell cultures persistently infected with SSPE-Kobe-1 were transfected with synthetic MV-L2 siRNA 6 h after cell seeding (coculture). After overnight incubation with siRNA, the cells were refed with fresh medium without containing siRNA and maintained in the culture to observe syncytium formation. Under this experimental condition too, SSPE virus replication in the infected cells was markedly inhibited by synthetic MV-L2 siRNA (Fig. 3C). Similarly, pcPUR + U6i-mediated MV-L2 siRNA brought about efficient inhibition of SSPE-Kobe-1 replication when the plasmid was transfected 6 h after cell seeding (Fig. 3D).

## 4. Discussion

The L protein of MeV is an RNA-dependent RNA polymerase that is essential for viral RNA replication, and yet it is least abundant among all the MeV proteins expressed (Barik, 2004; Lamb and Kolakofsky, 2001). Therefore, viral mRNA encoding the L protein would be a good target for the RNAi strategy to inhibit replication of MeV and SSPE virus. Indeed, we demonstrated in the present study that siRNAs targeted against selected portions of L mRNA of MeV, especially MV-L2 -L4 and -L5, either synthetic siRNAs or those expressed by pcPUR + U6i-based plasmids, effectively inhibited replication of both MeV and SSPE virus (Figs. 1–3). The inhibitory capacity of these otherwise effective siRNAs became barely detectable, if any, when the siRNAs were transfected to the cells 24 h after MeV infection (Fig. 1B). This result suggests that, once virus replication reaches its maximal level, viral mRNA molecules have accumulated abundantly in the cells to a level beyond the inhibitory capacity of siRNA. This idea might discourage an attempt to use siRNA for therapeutic purposes. However, it should also be stated that siRNA renders neighboring, uninfected cells resistant to virus infection, preventing the viral spread to the neighboring cells. In fact, the spread of SSPE virus from persistently infected cells was efficiently blocked by MV-L2 siRNA (Fig. 3A and B).

It is also noteworthy that, even in the transient transfection system where siRNA expression plasmids were introduced to at most 50% of the cells, MeV replication was inhibited by >90%

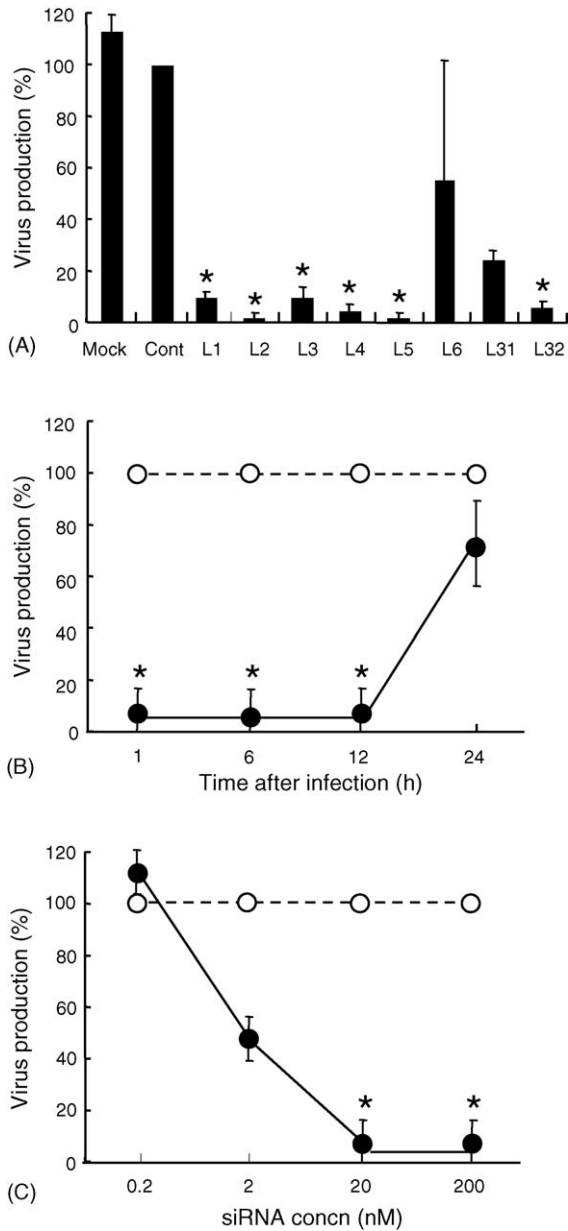


Fig. 1. Inhibition of MeV replication by synthetic siRNA. (A) Vero/SLAM cells were transfected with each of the synthetic siRNAs (20 nM) for 5 h and then inoculated with the K52 strain (genotype D3) of MeV. After 48 h, virus titers in the culture supernatants were determined. Synthetic NS3-2 siRNA (Takigawa et al., 2004) served as a control (Cont). Mock indicates cultures treated with the transfection reagent without siRNA. Data represent the mean  $\pm$  S.E.M. obtained from three independent experiments, which are expressed as the percentages of the number of syncytium seen in the wells treated with NS3-2 siRNA. \* $P < 0.05$ , compared with the control. (B) Cells were first infected with the virus, and after an indicated time, were transfected with 20 nM of MV-L2 siRNA (filled circle) or NS3-2 siRNA (open circle) for 5 h. Virus titers were determined 48 h postinfection. Data represent the mean  $\pm$  S.E.M. obtained from two independent experiments. \* $P < 0.01$ , compared with the control. (C) Cells were transfected with various concentrations (0.2, 2, 20 and 200 nM) of MV-L2 siRNA (filled circle) or NS3-2 siRNA (open circle) for 5 h and then inoculated with the virus. Virus titers were determined 48 h postinfection. Data represent the mean  $\pm$  S.E.M. obtained from two independent experiments. \* $P < 0.05$ , compared with the control.

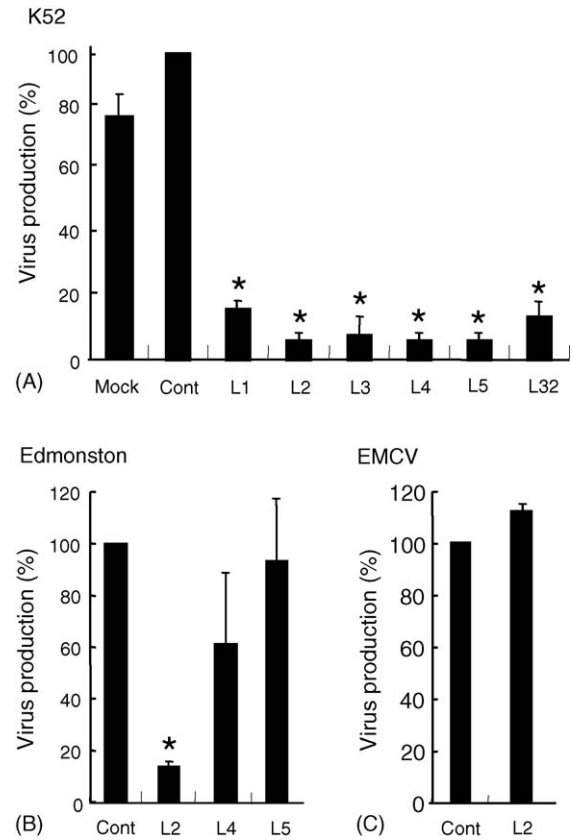


Fig. 2. Inhibition of MeV replication by plasmid-mediated siRNA. (A) Vero/SLAM cells were transfected with each of the pcPUR + U6i-based expression plasmids (2  $\mu$ g/well) overnight and then inoculated with the K52 strain of MeV. After 48 h, virus titers in the culture supernatants were determined. The NS3-2 siRNA-expressing plasmid served as a control (Cont). Mock indicates cultures treated with the transfection reagent without siRNA. Data represent the mean  $\pm$  S.E.M. obtained from two independent experiments, which are expressed as the percentages of the number of syncytium seen in the wells treated with the NS3-2 siRNA-expressing plasmid. \* $P < 0.01$ , compared with the control. (B) Vero/SLAM cells were transfected with each of the pcPUR + U6i-based expression plasmids (2  $\mu$ g/well) overnight and then inoculated with the Edmonston strains of MeV (genotype A). After 48 h, virus titers in the culture supernatants were determined. Data represent the mean  $\pm$  S.E.M. obtained from two independent experiments. \* $P < 0.01$ , compared with the control. (C) Vero/SLAM cells were transfected with each of the pcPUR + U6i-based expression plasmids (2  $\mu$ g/well) overnight and then inoculated with EMCV. After 48 h, virus titers in the culture supernatants were determined. Data represent the mean  $\pm$  S.E.M. obtained from two independent experiments.

by the siRNAs (Fig. 2 A and B). The most likely explanation for this remarkably efficient inhibition is that virus-infected cells form syncytia through fusion with their neighboring cells, at least some of which are expected to harbor the siRNA-expressing plasmid, and that de novo-produced siRNA is introduced to the virus-infected cells by virtue of syncytium formation. Although some siRNAs may activate the IFN system (Kim et al., 2004; Sledz et al., 2003), the siRNAs used in our study were unlikely to induce IFN activation since they did not inhibit replication of EMCV (Fig. 2C). EMCV is known to be highly sensitive to IFN (Song et al., 1999; Taguchi et al., 2004). This result also indicates that the siRNAs used in this study do not bring about any cytotoxic effect.



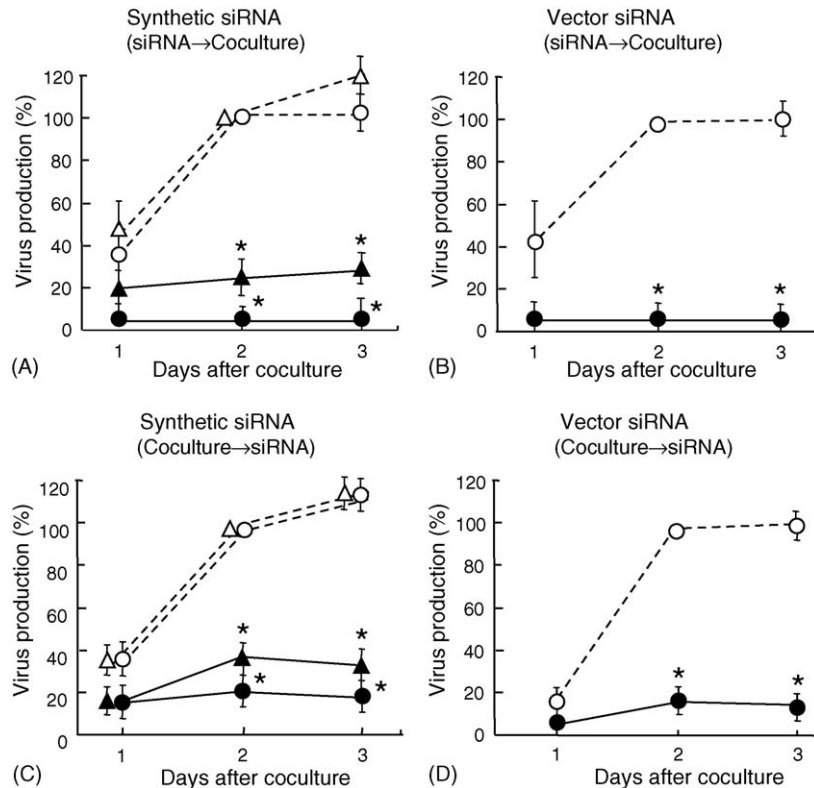


Fig. 3. Inhibition of SSPE virus replication by MV-L2 siRNA. (A) Vero/SLAM cells persistently infected with SSPE-Kobe-1 and fresh uninfected cells were each transfected overnight with 20 (filled triangle) and 200 nM of synthetic MV-L2 siRNA (filled circle), or 20 (open triangle) and 200 nM of synthetic NS3-2 siRNA (open circle). The virus-infected, siRNA-transfected cells were then cocultured with the uninfected, transfected cells. Syncytia that appeared on the monolayer cells were counted 24, 48 and 72 h thereafter. Data represent the mean  $\pm$  S.E.M. obtained from two independent experiments, which are expressed as the percentages of the number of syncytium seen in the wells treated with NS3-2 siRNA. \* $P < 0.01$ , compared with the control. (B) Vero/SLAM cells persistently infected with SSPE-Kobe-1 and uninfected cells were each transfected overnight with 2  $\mu$ g of pcPUR + U6i-MV-L2 siRNA (filled circle) or pcPUR + U6i-NS3-2 siRNA (open circle). The virus-infected, siRNA-transfected cells were then cocultured with the uninfected, transfected cells. Data represent the mean  $\pm$  S.E.M. obtained from two independent experiments. \* $P < 0.01$ , compared with the control. (C) Six hours after cell seeding, SSPE-Kobe-1-infected Vero/SLAM cells were transfected overnight with 20 (filled triangle) and 200 nM of synthetic MV-L2 siRNA (filled circle), or 20 (open triangle) and 200 nM of synthetic NS3-2 siRNA (open circle). Syncytia that appeared on the monolayer cells were counted 24, 48 and 72 h thereafter. Data represent the mean  $\pm$  S.E.M. obtained from two independent experiments. \* $P < 0.01$ , compared with the control. (D) Six hours after cell seeding, SSPE-Kobe-1-infected Vero/SLAM cells were transfected overnight with 2  $\mu$ g of pcPUR + U6i-MV-L2 siRNA (filled circle) or pcPUR + U6i-NS3-2 siRNA (open circle). Data represent the mean  $\pm$  S.E.M. obtained from two independent experiments. \* $P < 0.01$ , compared with the control.

Currently, there is no satisfactory specific treatment for SSPE and, therefore, the RNAi strategy might be considered as a potential therapeutic measure against this fatal disease. The sequence targeted by MV-L2 siRNA is well conserved across different genotypes of wild-type MeV and SSPE virus (data not shown). Considering its efficient silencing effect and the lack of cytotoxicity together, MV-L2 siRNA would be one of the good candidates for a clinical trial. However, a number of important issues should be addressed. It was recently reported that some viruses could escape RNAi by mutating the targeted viral sequences (Boden et al., 2003; Das et al., 2004; Gitlin et al., 2005; Leonard and Schaffer, 2005; Wilson and Richardson, 2005). Characteristic to an RNA virus, whose RNA-dependent RNA polymerase is error-prone, SSPE virus frequently undergo genetic mutations during its long-term persistence in a patient (Lamb and Kolakofsky, 2001). Therefore, a combined usage of multiple siRNAs that target different sequences would be recommended. At the same time, the possible off-target effect(s) of each siRNA should be examined thoroughly. We also need

to establish a good system to efficiently introduce siRNAs into the brain cells. We are currently generating recombinant adenovirus vectors expressing siRNAs against different portions of MeV/SSPE virus L mRNA. The usage of nonviral vectors, such as organically modified silica nanoparticles (Bharali et al., 2005), would be another promising option. In any case, the safety of the vectors when applied in vivo should be completely warranted.

In conclusion, we have developed siRNAs that can effectively inhibit replication of MeV and SSPE virus. Although a number of important issues should be addressed before its possible clinical application, these siRNAs may be a potential candidate for therapeutic measures against the currently incurable SSPE.

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