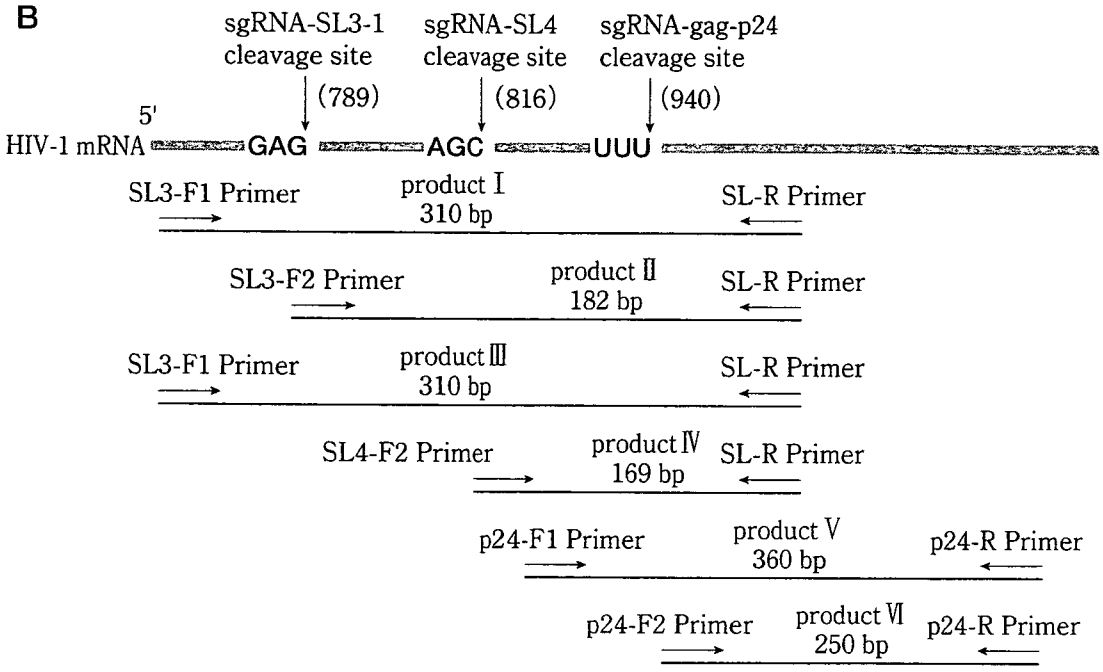

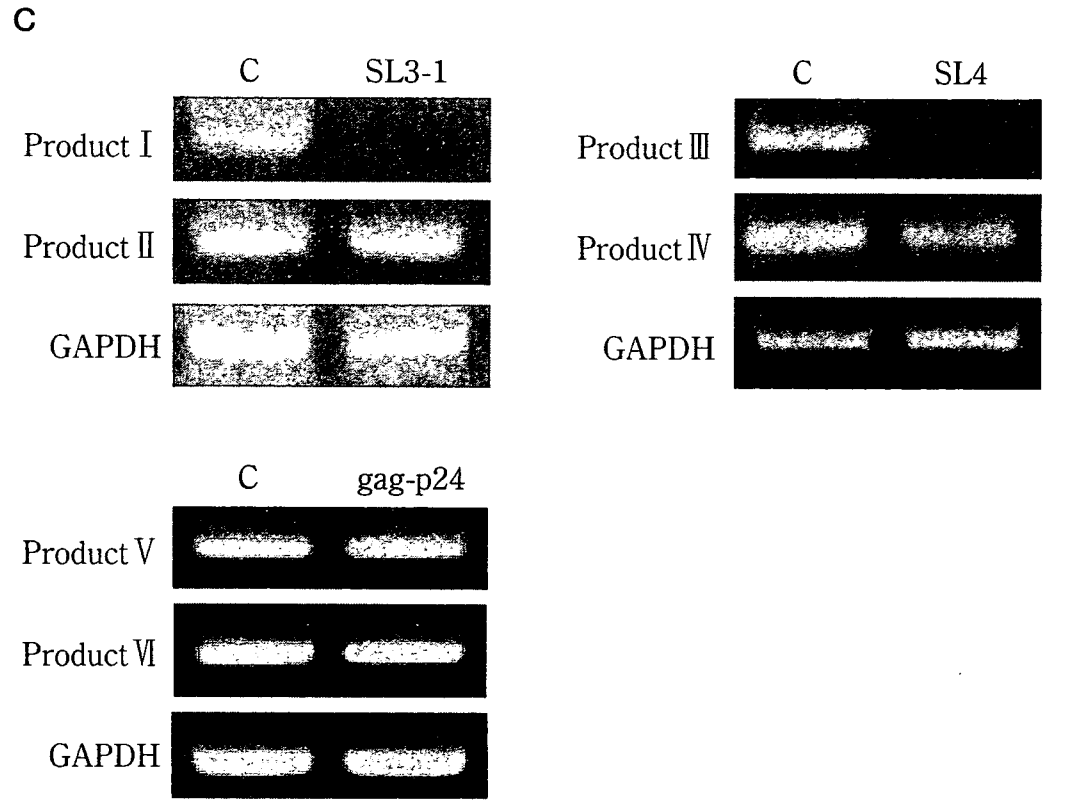

図4

B：RNase Z による標的 RNA の切断を RT-PCR 法にて標的 RNA を検出するためのプライマー設定部位。

C：プロダクト別の RNA 検出結果

を抑制することから、RNase Pの認識能には多様性があることが示唆された。

7. tRNase ZLによるウイルス遺伝子の発現制御

次に、われわれがtRNase ZLを利用してHIV-1の発現を制御した例を紹介する[27,28]。

tRNase ZLは、small-guide RNA（sgRNA）と標的RNAがつくるtRNA前駆体様の構造を認識して標的RNAの3'末端側を切断する機能をもっている。この機能を利用してHIV-1遺伝子を切断するために、sgRNAを発現するプラスミドベクターを構築し、ウイルス感染標的細胞に導入して、ウイルス遺伝子を切断するか否かを検討した。ここで標的としたウイルス遺伝子は、sgRNAが結合したときにtRNA前駆体様の構造をとることが予測される5'末端側LTR領域と、構造遺伝子をコードする*gag*遺伝子領域の一部分（p24領域）とした。これらの遺伝子に結合するsgRNAを発現するベクターを、野生変異型ウイルスベクターにレポータ遺伝子を組み込んだpNL-lucベクターとともに細胞

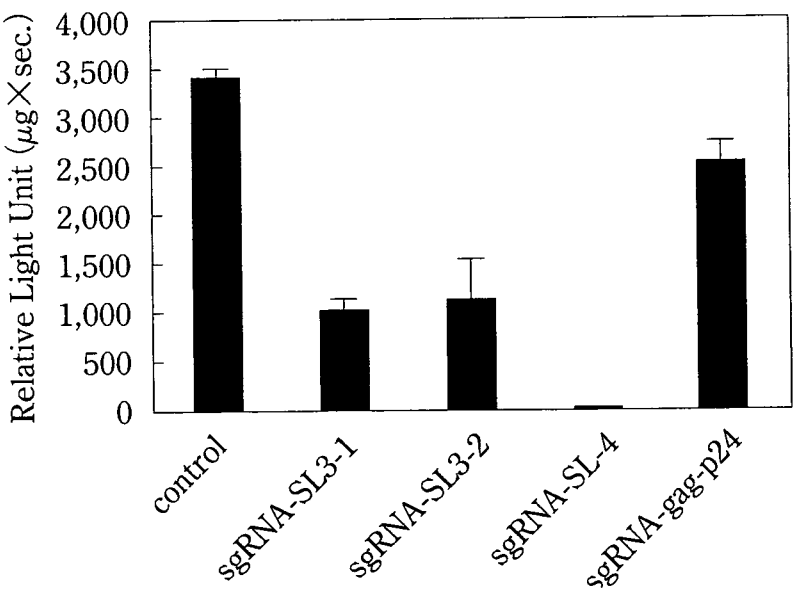

図5　ウイルス由来たんぱく質の発現量を定量

左端のコントロールベクター導入サンプルと比較してsgRNA導入細胞（SL-4）にウイルス由来たんぱく質の発現を強く抑制した

に導入し、ウイルス由来のたんぱく質発現量を測定した。その結果、sgRNAの標的遺伝子によってウイルス発現制御に違いがみられた（**図4**）。同時に、ウイルスRNAの切断についても確認したところ、その活性はsgRNAの標的遺伝子配列に左右されることが示唆された（**図5**）。また、tRNase ZLによるウイルス遺伝子の切断は、ウイルスたんぱく質の発現を2週間以上にわたって抑制することも確認された。

以上のことから、sgRNAの標的遺伝子の配列ならびに二次構造は、tRNase ZLのRNA切断能に重要であることが示唆された[29]。

おわりに

現在、ウイルス感染症に対する治療法の主力である抗ウイルス剤は、薬剤耐性ウイルスの出現や薬剤間の交差耐性などによる有効性の減少が問題となっている。また、副作用も非常に強いことから、ウイルス遺伝子に特異的に働く薬剤（物質）の検索が急務とされている。本稿で紹介した生体内RNAプロセシング機構を利用したウイルス発現制御法は、これらの問題を解決することができるのではないかと期待される。

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4 Suppression of HIV-1 replication by shRNA

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Abstract

The degradation of a selected mRNA species by RNA interference requires a high degree of homology between the short interfering or short hairpin RNA (si or shRNA) and its target. Short interfering RNAs (siRNA) are widely used for targeting and silencing genes by RNA interference. The recent discovery that exogenously delivered shRNA can trigger RNAi in mammalian cells allows the use of this technology in research and perhaps eventually as a therapeutic tool. Inhibition of HIV-1 replication with RNAi has already been demonstrated with siRNAs against a variety of structural and regulatory genes including gag, pol,

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nef, vif, tat, env and rev. These results revealed that siRNA directed to an HIV-1-specific gene could inhibit viral replication. In addition to targeting viral genes, many studies have also investigated the efficacy of siRNAs in down-regulating the host cell molecules necessary for HIV-1 infection. However, the induction of IFN-β by siRNA proceeds through the activation of a number of cellular transcription factors, the most important of which are NF-κB and IRF-3. We found that the position of the 5'-overhang influenced the inhibition of IFN-β expression.

Introduction

RNA interference (RNAi) or RNA silencing is an evolutionarily conserved phenomenon in which double-stranded RNA (dsRNA) induces the homology-dependent degradation of the cognate mRNA.[1] RNAi is initiated by the RNase III-like nuclease Dicer, which promotes the processive cleavage of long dsRNAs into 21- to 23-nucleotide (nt) small interfering RNAs (siRNAs) with 2-nt 3' overhangs.[2-6] Subsequently, the siRNAs are incorporated into an RNA-induced silencing complex (RISC), identified in *Drosophila*, and the protein-RNA effector nuclease complex recognizes and destroys the target mRNAs.[7-9]

It was recently found that the transfection of synthetic 21-23 nt siRNAs with 2-nt 3' overhangs into mammalian cells effectively inhibits the expression of the endogenous genes in a sequence-specific manner.[10-12] These small RNA duplexes, which are chemically synthesized mimics of Dicer products, are presumably incorporated into RISC and target their cognate substrates for degradation. Lee *et al.* have reported the inhibition of human immunodeficiency virus type 1 (HIV-1) replication by the expression of small interfering RNAs targeted against HIV-1 *rev* in human cells.[13] In addition, Jacque *et al.* demonstrated the utility of siRNA-mediated RNAi for modulating HIV replication by synthetic siRNAs or plasmid-derived siRNAs targeted to various regions of the HIV-1 genome (TAR, *vif, nef*).[14]

We previously reported that long dsRNAs effectively inhibit HIV-1 replication in HIV-1 infected cells.[15] However, the utility of these long dsRNAs appeared to be limited, due to the nonspecific inhibitory response resulting from the activation of a dsRNA-dependent protein kinase (PKR) and a 2'-5'-oligoadenylate (2-5A) synthetase.[16,17] A 21-23 nt siRNA can mediate RNAi and bypass the non-specific response induced by longer dsRNAs.

Design of RNAi

In this study, we have designed four siRNAs against several regions of the HIV-1 *env* genes, to test a more useful antiviral RNAi technology. The mRNA targets for siRNAi were selected from the middle of the *env* regions in the HIV-1 genome, since we previously found that 531 bp (7070~7600) E2-dsRNAs,

complementary to the *env* mRNA-containing V3 loop and the major CD4 binding domain sequence of gp120, were more effective inhibitors of HIV-1 replication than those targeted to the *gag* gene. [15] Furthermore, the envelope protein (Env) of HIV-1 mediates functions that are critical to the viral life cycle, including the viral attachment to target cells and the fusion of viral and cellular membranes.

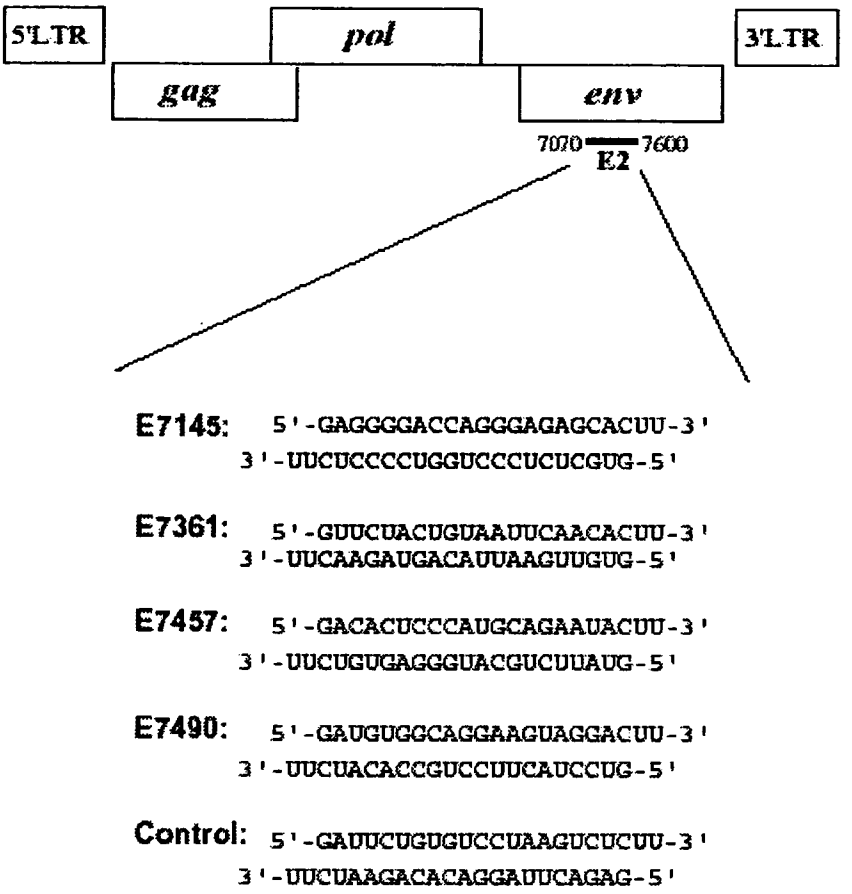

Figure 1. The target sites and the sequences of the siRNAs used in this study. The sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting the HIV-1_{NL4-3} *env* gene are shown. The siRNA duplexes are composed of 22-nucleotides (nt) with 2-nt ribo-uridine 3' overhangs, and are numbered according to the position of the first nucleotide of the sense strand. The 18 nt sense strand, corresponding to six functional amino acids, is completely homologous to the target HIV-1 sequence. To generate siRNAs *in vitro*, we designed DNA template oligonucleotides with a T7 promoter sequence. The templates corresponding to the sense and antisense sequences of the target gene were mixed in equimolar amounts, heated to 95°C for 5 min, and then gradually cooled to room temperature in TE buffer (10 mM Tris [pH8.0], 0.1 mM EDTA). *In vitro* transcription was carried out by using the AmpliScribe Transcription Kit (Epicentre Technologies, Wisconsin, USA) with 1 μg of DNA template. Following the transcription reaction, equimolar quantities of the sense and antisense 22-nt RNAs generated in separate reactions were annealed by mixing and heating at 95°C for 5 min, followed by a 1 h incubation at 37°C to obtain the T7 siRNA.

To investigate the siRNA-mediated silencing, we initially synthesized four siRNAs against the middle of the *env* regions in the HIV-1 genome (Fig. 1). For *in vitro* transcription, DNA template oligonucleotides with a T7 promoter sequence (TAATACGACTCACTATAG) were designed to produce 22-nt siRNAs. The siRNA sequences of the form $GN_{18}CN_2$ were selected for each target, because efficient T7 RNA polymerase initiation requires a G as the first nt of each RNA.[18] Sense and antisense RNAs were synthesized with T7 RNA polymerase separately *in vitro* from a dsDNA template, and were annealed to each other to create a 22-nt siRNA duplex with 2-nt ribo-uridine 3' overhangs at each end.

Suppression of HIV-1 activity

To test whether siRNAs can specifically inhibit HIV-1 gene expression, we co-transfected COS cells with HIV-1 proviral expression plasmids (pNL4-3)[19] and either a synthetic siRNA duplex or a single-stranded RNA corresponding to HIV-1 *env* (Figure 2a). Examination of the cellular uptake of the fluorescently-labeled-siRNAs, prepared with a Silencer™ siRNA Labeling kit (Ambion, Texas, USA), with several kinds of transfection reagents (Lipofectamine™ 2000[12,18], Lipofectin, and Oligofectamine) by the FACS Calibur and CellQuest software, revealed that the FITC-labeled-siRNAs encapsulated with Lipofectamine™ 2000 strongly associated with the target cells (data not shown). Co-transfection of the plasmid (pNL4-3) and siRNAs was carried out using Lipofectamine™ 2000. The virus production in the culture supernatant was assessed by an HIV-1 p24 CLEIA assay[20] at three days, and was related to the amount produced in the absence of the siRNA. The four *env*-specific siRNAs all effectively inhibited HIV-1 gene expression, with a wide range of activities. In cells transfected with E7145, targeted to the central region of the V3 loop, or E7490, targeted to the CD4 binding site of conserved regions on gp120, the p24 antigen expression was reduced to near background levels, as shown in Figure 2a. Interestingly, the inhibition mediated by the siRNA E7457 was higher than that seen with the siRNA E7361 in the targeting of an intersubunit disulfide bond on gp120. The sense RNAs were ineffective in reducing the amount of p24 antigen, whereas the antisense RNAs modestly reduced the amount of p24 antigen. However, the four siRNAs directed against HIV-1 *env* effectively inhibited HIV-1 gene expression by more than ~1.5-fold, relative to the antisense RNAs. Importantly, the specificity of this effect was demonstrated by the finding that a nonhomologous control siRNA had no significant effect on HIV-1 suppression.

To characterize the siRNA-mediated RNAi reaction further, we tested the dose-dependent effect of the siRNAs. COS cells were co-transfected with

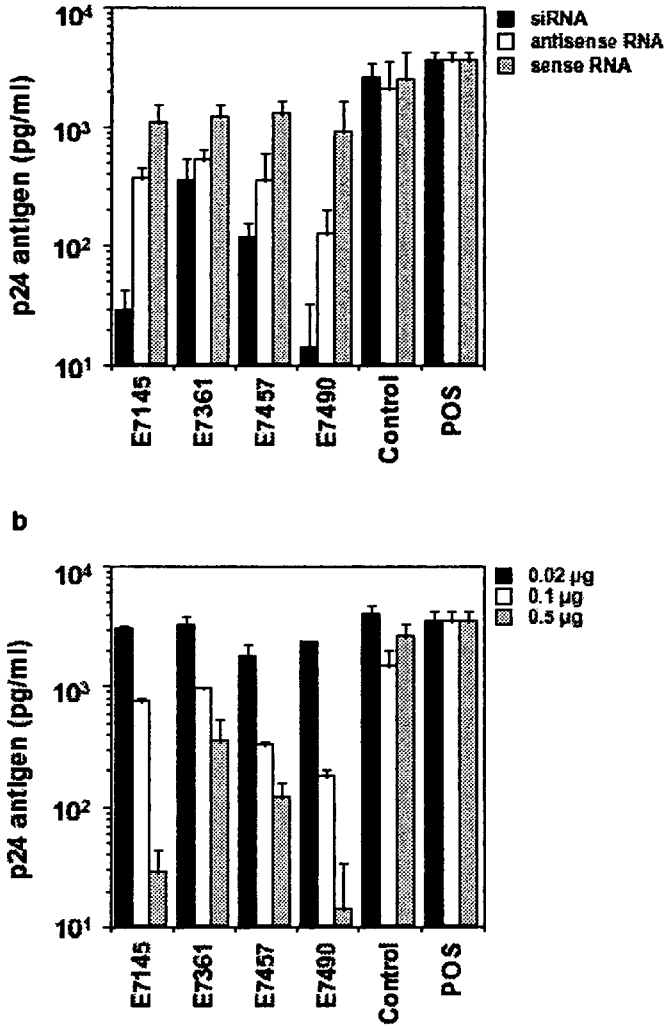

Figure 2. Specific inhibition effects by siRNAs directed against HIV-1 *env*. (a) Inhibitory effects of siRNA duplexes, sense and antisense RNAs on HIV-1 gene expression. Twenty-four h before transfection, COS cells were seeded in 35-mm dishes at a density of 10^5 cells. Transient transfection of siRNAs was carried out using Lipofectamine™ 2000 (Invitrogen, Groningen, Netherlands). A 48 µl aliquot of RPMI-1640 medium without serum was mixed with 2 µl of Lipofectamine™ 2000 per dish, and the mixture was preincubated for 5 min at room temperature. During this incubation, another 50 µl aliquot of RPMI-1640 medium was mixed with pNL4-3 (0.1 µg) and the various single-stranded or double-stranded siRNAs (0.5 µg) as indicated. The two mixtures were combined and incubated for 20 min at room temperature for complex formation. The entire mixture was then added to the cells. The plasmid pNL4-3 is an HIV-based infectious vector, which was purified with the plasmid Maxi-prep Kit (SIGMA, St. Louis, USA), followed by phenol extraction and ethanol precipitation. At three days post transfection, p24 Gag protein production was detected by the HIV-1 p24 CLEIA assay (Lumipulse; Fujirebio Inc., Tokyo, Japan). (b) Dose-dependence of *env*-specific siRNAs. COS cells were co-transfected with pNL4-3 (0.1 µg) and various amounts of siRNAs (0.02-0.5 µg), using Lipofectamine™ 2000. After a 72 h incubation, p24 antigen production was monitored in the culture supernatants with the HIV-1 p24 CLEIA assay. Mock-transfected COS cells served as the positive control (POS).

various concentrations (0.02-0.5 μg) of the siRNAs and pNL4-3, using Lipofectamine™ 2000 (Fig. 2b). The control siRNA did not induce any significant suppression of HIV-1 gene expression in this dose-dependent assay. The four *env*-specific siRNAs induced the RNA interference-mediated HIV-1 inhibition in a dose-responsive manner. In particular, 0.5 μg of E7490 showed a significantly high inhibitory effect on HIV-1 gene expression. Therefore, these results suggest that siRNAs inhibit HIV-1 gene expression in a sequence-specific manner.

We next examined the inhibition of HIV-1 gene expression at the mRNA level, to identify the contribution of the siRNA-mediated specific RNA interference. Total RNA was purified from COS cells that had been transfected with the four homologous siRNAs or the nonhomologous control siRNAs and pNL4-3, and equal amounts of RNA were subjected to a polymerase chain reaction with RT-PCR analysis. The treatment with either of the two best siRNAs, E7145 or E7490, induced a marked reduction in the levels of HIV-1 mRNA relative to the levels in cells that were transfected with control siRNA or plasmid-only. Therefore, the siRNA-mediated RNAi is fully functional and sequence-specifically inhibits HIV-1 gene expression (data not shown).

Next, to clarify the ability of siRNA to inhibit viral replication in HIV-1 infected cells, we first used the HeLa-CD4$^+$ cell line. Sense, antisense, and siRNAs (0.5 μg), encapsulated with Lipofectamine™ 2000, were each transfected into HeLa-CD4$^+$ cells. Following a 4 h incubation at 37°C, the cells were inoculated with the HIV-1_{NL4-3} virus. As shown in Figure 3, the two best siRNAs, E7145 and E7490, significantly inhibited HIV-1 replication, similar to the level obtained with the COS cell-based assay. The four siRNAs targeted to the HIV-1 *env* genes effectively inhibited HIV-1 replication more than ~2-fold relative to the antisense RNAs. In contrast, the four sense RNAs showed only slight suppression of the p24 expression. These results indicate that the four siRNAs targeted to the HIV-1 *env* genes have potent and specific inhibitory effects on HIV-1 replication. They also suggest that the siRNA is more effective in targeting the HIV-1 genes than the antisense RNA.

In addition, we examined whether the siRNAs could inhibit HIV-1 replication in human peripheral blood mononuclear cells (PBMCs), which are natural targets for HIV-1 infection. The *env*-specific siRNA inhibitory effect and its persistence on viral replication were investigated in HIV-1 infected PBMCs over a 14 day period. PHA-stimulated PBMCs were infected with the HIV-1_{NL4-3} virus, in the presence of the siRNAs E7145 and E7490. In the cells treated with the *env*-specific siRNAs, E7145 and E7490, the p24 antigen expression was effectively reduced as compared to that in the untreated cells (Fig. 4a). Moreover, E7145 and E7490 were each effective against HIV-1_{NL4-3} replication for a relatively long time (14 days). Next, to assess the effects of the single-stranded RNA and the siRNA duplexes in PBMCs, we compared the

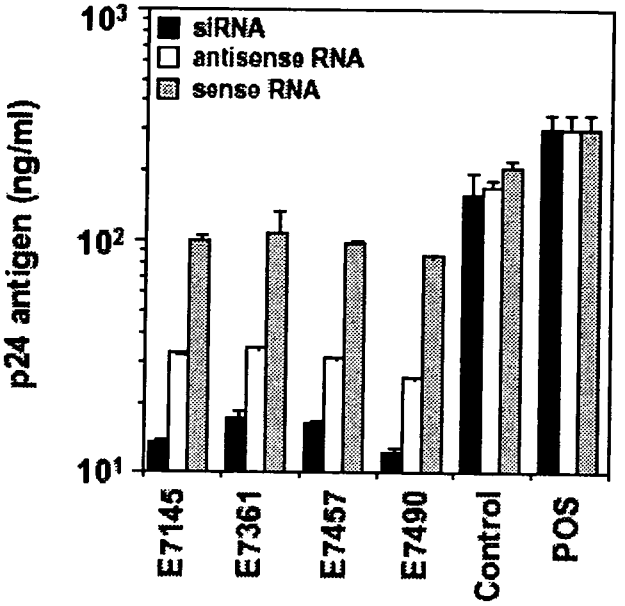

Figure 3. siRNA-mediated inhibition of HIV-1 replication in HIV-1 infected HeLa-CD4$^+$ cells. HeLa-CD4$^+$ cells ($2x10^5$ cells) were seeded into 6-multiwell plates 24 h before transfection, and then were transfected with the various single-stranded or double-stranded siRNAs (0.5 μg), using Lipofectamine^{TM} 2000. After a 4 h incubation, the transfected cells were infected with HIV-1_{NL4-3} virus at a multiplicity of infection (MOI) of 0.5. HeLa-CD4$^+$ cells were transfected at approximately 48 h intervals, and were asssayed 7 days after the first transfection by the HIV-1 p24 CLEIA assay. HeLa-CD4$^+$ cells infected in the absence of siRNAs served as the positive control (POS).

inhibitory effects of siRNAs targeted to the HIV-1 *env* gene with those of the corresponding sense and antisense RNAs at 10 days post infection (Fig. 4b). The siRNA duplexes directed against the HIV-1 *env* genes more effectively inhibited HIV-1 replication relative to the single-stranded RNA. In addition, the siRNAs E7145 and E7490 directed against *env* reduced the p24 antigen levels in a dose-dependent manner (data not shown). Taken together, our results indicate that the siRNA-mediated RNAi is fully functional in cells naturally targeted by HIV-1 infection.

In this report, we have shown that the synthetic siRNAs targeted to the HIV-1 *env* gene can effectively and specifically inhibit HIV-1 gene expression by reducing the viral mRNA expression. Furthermore, the siRNA duplexes were more potent inhibitors than the antisense RNAs. Our best siRNA candidates, E7145 targeted to the central region of the V3 loop and E7490 targeted to the CD4 binding site of the conserved regions on gp120, significantly inhibited the HIV-1 gene expression. Especially, E7145 and E7490 were effective against HIV-1_{NL4-3} replication in PBMCs for a relatively long time (14 days)[21]. Therefore, we suggest that siRNA-mediated RNA interference, targeted to the HIV-1 *env* gene, can be developed as a new potential therapeutic strategy for AIDS.

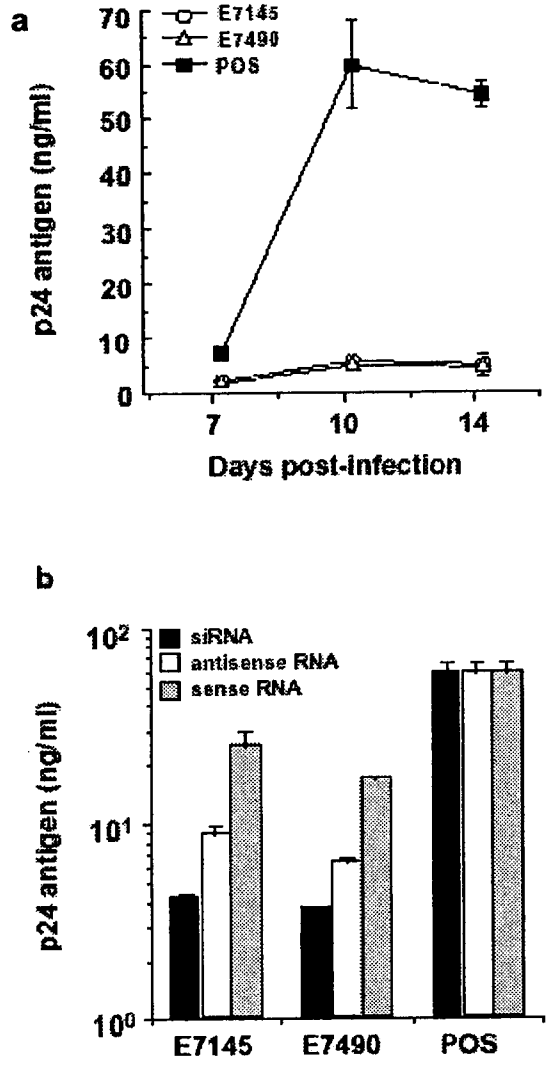

Figure 4. Effects of siRNAs directed against HIV-1 *env* in HIV-1 infected PBMCs. (a) Time course of siRNA-mediated RNAi. Human peripheral blood mononuclear cells (PBMCs) from healthy HIV-1 negative donors were isolated by Ficoll-Hypaque gradient centrifugation, grown in RPMI-1640 medium supplemented with 10% FBS, and activated with 1 μg/ml of phytohemagglutinin (PHA; Seikagaku Corp., Tokyo, Japan) for 3 days in the presence of IL-2 (100 U/ml; Shionogi, Osaka, Japan) at 37°C. Three days later, the PHA-stimulated PBMCs (10^6 cells) were infected with the HIV-1_{NL4-3} virus at an MOI of 0.001 in the presence of the encapsulated E7145 or E7490 siRNA (0.5 μg). At 7, 10 or 14 days after infection, culture supernatants containing the progeny viruses were removed, and the amount of p24 was measured by an HIV-1 p24 CLEIA assay. Fresh medium was then added with one-half volume of culture medium, and the cells were transfected with each of the indicated siRNAs (0.5 μg), using Lipofectamine™ 2000. (b) Comparison of the effect of sense, antisense, and siRNAs directed against HIV-1 *env*. The PHA-stimulated PBMCs were transfected with the sense, antisense or siRNAs (0.5 μg) encapsulated with Lipofectamine™ 2000, and were infected with HIV-1_{NL4-3} at an MOI of 0.001 after 4 h. At 10 days post infection, progeny virus production was measured by the HIV-1 p24 CLEIA assay. PBMCs infected in the absence of siRNAs served as the positive control (POS).

In particular, the use of T7 RNA polymerase-synthesized siRNA to obtain large amounts of siRNAs offers the advantages of simplicity and economical efficiency. These approaches will be crucial for overcoming the limited availability of synthetic siRNA technology for gene silencing in mammalian cells; furthermore, they will be useful for extending the potential of siRNA to therapies for HIV infection. However, aside from the advantages offered by siRNA for viral infection control, bottlenecks, including the dosages for actual clinical situations, could challenge future researchers in the clinical field. In spite of that, the simplicity of siRNA-mediated RNAi technology may enable 'combination therapy' along with several siRNAs targeted to different regions of *env* or HIV-1-specific genes. They could constitute a new approach toward preventing the emergence of drug-resistant viruses. Moreover, further development and modifications of viral vector-based systems capable of expressing active *env*-siRNA will assist in the establishment of effective clinical applications against HIV-1.

Side-reaction of cytokine by RNAi

The degradation of a selected mRNA species by RNA interference requires a high degree of homology between the si or shRNA and its target. siRNAs are widely used for targeting and silencing genes by RNA interference. The recent discovery that exogenously delivered shRNA can trigger RNAi in mammalian cells has promoted the use of this technology in research, and perhaps it will eventually become a therapeutic tool. The inhibition of HIV-1 replication with RNAi has already been demonstrated with siRNAs against a variety of structural and regulatory genes including *gag*, *pol*, *nef*, *vif*, *tat*, *env* and *rev*. These results demonstrate that siRNA directed to an HIV-1-specific gene could inhibit viral replication. In addition to targeting viral genes, many studies have also investigated the efficacy of siRNAs in down-regulating the host cell molecules necessary for HIV-1 infection. However, the induction of IFN-β by siRNA proceeds through the activation of a number of cellular transcription factors, the most important of which are NF-κB and IRF-3. We found that the position of the 5'-overhang affected the expression of IFN-β.

We confirmed that IFN-β induction can be avoided by using pppGG-shRNAs or pppGGG-shRNs synthesized by T7 RNA polymerase. In contrast, the IFN response was initiated slightly by the pppGn (n=1) associated with the 5' end of shRNA (data not shown). Furthermore, the pppGn (n=0) associated with the 5' end of shRNA showed more potent IFN-β induction than the pppGn (n=1) associated with the 5' end of shRNA. In addition, non- IFN-β induction in HeLa $CD4^+$ cells was elicited by the 5'-HOGn (n=0-3).

We also observed the anti-HIV-1 activity of pppGG-shRNADIS. From these analyses we concluded that the residual amount of shRNA containing 5'-G was proportional to the reduction of the IFN-β response. The pppGG-shRNADIS showed sequence-specific suppression of gag p24 production. These results suggest that the nature of the 5' end of the shRNAs must be considered to avoid IFN-β induction.

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7 RNAi and SARS

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Abstract

Outbreaks of severe acute respiratory syndrome (SARS), caused by the newly identified coronavirus SARS-CoV, occurred between 2002 and 2003. A number of prophylactic and therapeutic strategies for SARS were reported but specific treatment for this disease has not been established yet. RNA interference (RNAi) is triggered by the presence of a double-stranded RNA (dsRNA), and results in specific degradation of mRNA containing the same sequence. Recently it has been shown that siRNA can inhibit replication of various kinds of viruses including SARS-CoV in vitro and in vivo. This review will first provide current information on SARS-CoV and virus-targeting siRNA, and will then discuss improvement of RNAi technology for efficient and stable silencing.

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Introduction

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is a novel coronavirus identified in late March 2003, and the WHO announced on April 16 that this coronavirus was the definitive cause of SARS [1-4]. SARS represents typical influenza-like symptoms such as high fever, myalgia, dyspnea, lymphopenia and pneumonia, with the severe acute breathing problems which raise the overall mortality to 10%. A total of 8098 cases of SARS were reported from 26 countries, and 774 patients died as a result between 1 November 2002 and 31 July 2003 [5].

The outbreak of SARS has posed a necessity to develop strategies to deal with emerging infectious diseases. Certain empirical measures such as antibiotics, antiviral agents, corticosteroids, and interferons were applied to treat patients suffering from SARS [6-9], but no specific and effective strategy has not been established yet. Researchers have made efforts to find new therapeutic strategies and tested many kinds of treatments including RNA-mediated interference (RNAi) [10-19].

RNAi was originally discovered in plants and then found in other organisms such as Caenorhabditis elegans, Drosophila, and vertebrates[20-22]. It is an evolutionarily conserved process for suppression of gene expression[21, 23-25] . In this process, recognition of double-stranded RNA leads to the production of small interfering RNAs (siRNAs) of 21 to 22 nucleotides (nt), which associate with a multiprotein complex known as the RNA-induced silencing complex and ultimately target homologous mRNA for degradation based on complementary base pairing.

RNAi has been successfully used in blocking the replication of some viruses such as human immunodeficiency virus and human hepatitis C virus[26-30]. Since SARS-CoV is an RNA virus, RNAi could be a reasonable approach for therapeutic purposes against SARS.

In this chapter, the current information on SARS-CoV and RNAi will be reviewed and RNAi as a therapeutic strategy against SARS will be discussed.

Genome organization of SARS-CoV

Coronaviruses, a genus of the coronaviridae family, are large, enveloped, positive-stranded RNA viruses, and can cause many infectious diseases in humans and animals[31, 32]. Before the SARS outbreak, coronaviruses consisted of three groups. Group I includes human HCoV-229E, porcine transmissible gastroenteritis virus (TGEV), whereas HCoV-OC43, mouse hepatitis virus (MHV) and bovine coronavirus (BCoV) belong to group II. Group III includes avian infectious bronchitis virus (IBV) and turkey coronavirus (TCoV).

Sequence analysis of the entire SARS-CoV RNA genome indicated that the virus genome was very similar to previously characterized coronaviruses, with the same order of the genes (replicase (R), spike (S), envelope (E), membrane (M) and nucleocapsid (N) gene), where there are few accessory genes or motifs spanning between the structural genes and at the 3' UTR(untranslated region) (Fig. 1 and table 1) [2, 33-35].

All coronaviruses have a very similar organization in their functional and structural genes, but the arrangement of the non-essential genes is notably different among the subgroups. Group I coronaviruses are chiefly characterized by the presence of ORFs following the N gene. Group II coronaviruses include two additional ORFs, non-structural protein 2 (ns2) and HE gene, located between ORF 1b and the S gene. Only group III coronaviruses have ORFs between the M and N gene, and a conserved stem-loop motif s2m at their 3' UTR (Fig. 1 and table 1). Accessory ORFs are found between the S and E genes in all of the subgroups.

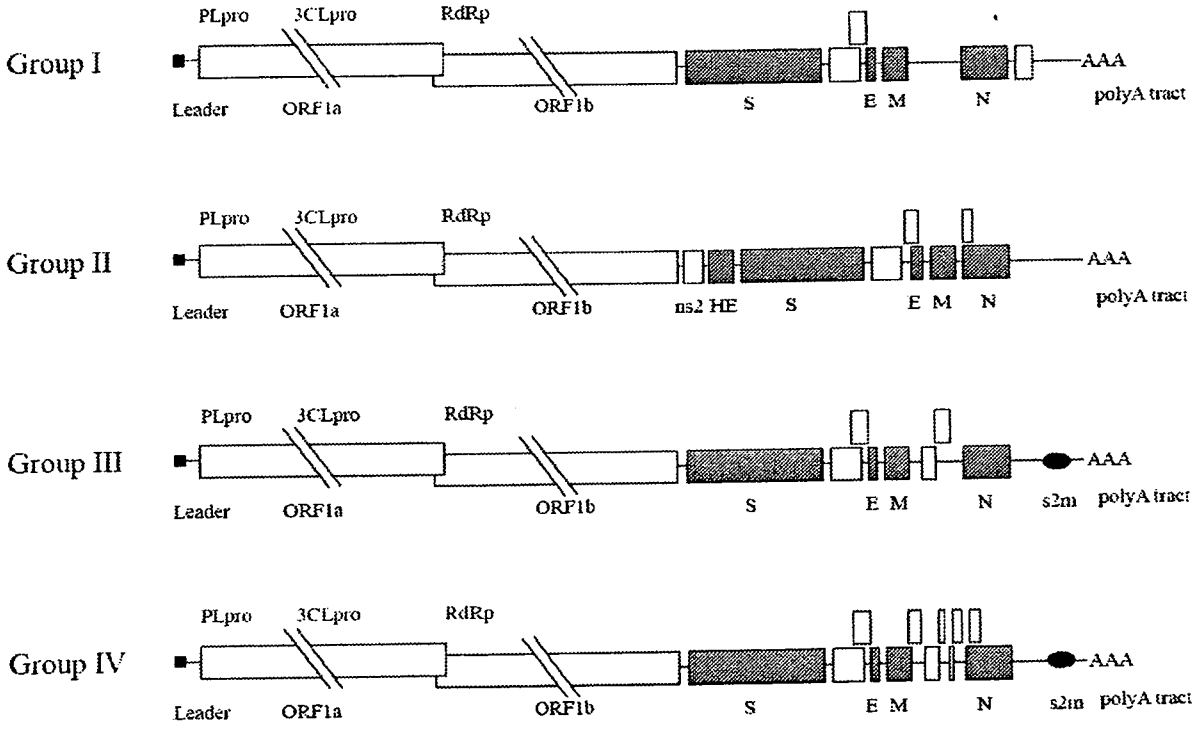

Figure 1. Gene organization of coronaviruses. Coronaviruses are divided into 4 classes on the basis of their genome organization. SARS-CoV represents a new class of coronavirus, group IV. The boxes indicate ORFs and shaded boxes are coding sequences for structural proteins. PLpro: papain-like cysteine protease; 3CLpro: 3C-like cysteine protease; RdRp: RNA-dependent RNA polymerase; HE: Haemagglutinin esterase; S: Spike protein; E: Envelope protein; M: Matrix protein; N: Nucleocapsid protein.

Table 1. Features of SARS-CoV genome sequence and subgenomic transcripts.

mRNA	ORF				Start	End	No. of a.a.	No. of Bases	Frame
	Marra et al.	Rota et al.	Thiel et al.	Zeng et al.					
mRNA1	ORF 1a	ORF 1a	ORF 1a	ORF 1a	265	13398	4382	13149	+1
mRNA1	ORF 1b	ORF 1b	ORF 1b	ORF 1b	13398	21485	2628	7887	+3
mRNA2	S protein	S protein	S protein	S protein	21492	25259	1255	3768	+3
mRNA3	ORF 3	X1	ORF 3a	X1	25268	26092	274	825	+2
mRNA3	ORF 4	X2	ORF 3b	N/R	25689	26153	154	465	+3
mRNA4	E protein	E protein	E protein	N/R	26117	26347	76	231	+2
mRNA5	M protein	M protein	M protein	M protein	26398	27063	221	666	+1
mRNA6	ORF7	X3	ORF6	N/R	27074	27265	63	192	+2
mRNA7	ORF 8	X4	ORF 7a	X2	27273	27641	122	369	+3
mRNA7	ORF 9	N/R	ORF 7b	N/R	27638	27772	44	135	+2
mRNA8	ORF 10	N/R	ORF 8a	X3	27779	27898	39	120	+2
mRNA8	ORF 11	X5	ORF 8b	N/R	27864	28118	84	255	+3
mRNA9	N protein	N protein	N protein	N protein	28120	29388	422	1269	+1
mRNA9	ORF 13	N/R	ORF 9b	N/R	28130	28426	98	297	+2

Pairwise sequence homology search among the accessory ORFs at the S-E intergenic region of the SARS-CoV (ORF3a,b) and all other coronaviruses shows no significant sequence homology [33, 34, 36] but they are homologous within subgroups. The ORF 5a/5b of group III coronaviruses and ORFs 6-8 of the SARS-CoV are located in a homologous region, but they do not show any significant sequence homology.

The phylogenetic analysis by the comparison of the deduced amino acid sequences of the replicase gene and four structural genes (S, E, M, N) with other coronaviruses was performed [33, 34, 36]. The conclusions drawn by the different research groups were similar, with the observation that SARS-CoV itself forms a distinct cluster, the fourth group of Coronaviridae. Moreover, no detectable recombination event was concluded in the similarity plot on the whole genome alignment with other coronaviruses [36]. The divergence of ORFs between the SARS-CoV and other known coronaviruses suggests that the SARS-CoV might have been circulating in other animal hosts long before its emergence and somehow crossed into a human host by a sudden mutation event or a RNA recombination event with unknown sources.

Infection of SARS-CoV and replication of its genome

Virus entry

The entry of coronaviruses involves three steps, namely, attachment, receptor binding and virus-cell fusion, which are mediated by viral envelope proteins (Fig. 2). The S glycoproteins, which form large petal-shaped spikes on the surface of the virion, have a molecular mass of 150-180 kDa and can be divided into three structural domains: a large external N-terminal domain (consisting of subdomains S1 and S2), a transmembrane domain and a short

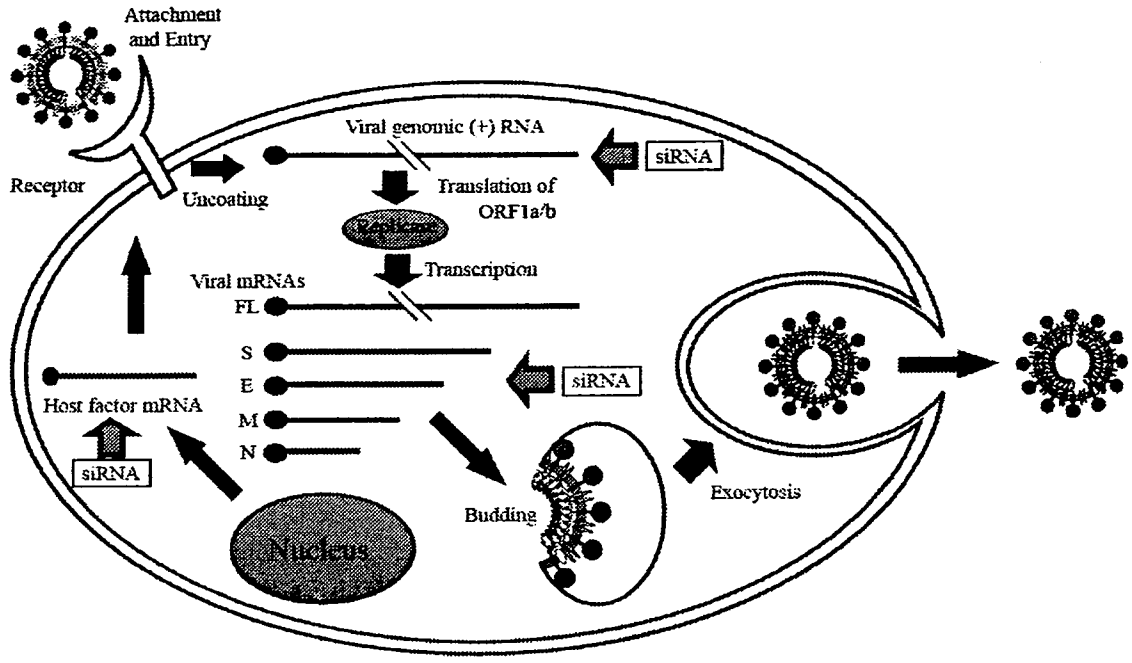

Figure 2. Replication of SARS-CoV and potential targets of siRNA. The life cycle of SARS-CoV starts with the interaction between S protein and the cellular receptor. Silencing the virus receptor may prevent entry of the virus and S protein may also be a good target for RNAi. The RNA genome of the virion is released into the cytoplasm after attachment of the virion to the host receptor, and the virus produces polyproteins encoding ORF1a and 1b by using the translational machinery in host cells. Cleavage by virally encoded proteases yields the components required for assembling the viral replicase complex. These components in replicase complex including RNA-dependent RNA polymerase are potential siRNA targets. In addition to the viral genomic RNA, the subgenomic RNAs encoding E, M and N may be degraded by siRNAs.

C-terminal cytoplasmic domain[37]. The receptor for SARS-CoV is considered to be angiotensin-converting enzyme 2 (ACE2), which is essential in binding of S1 subdomain to the surface of permissive cells[38]. Fine mapping on the N-terminal unit of the spike protein indicates that the receptor-binding domain is probably located between the residues 303 and 537 [39].

Replicase gene

After the step of virus entry, large polyproteins (ORF1a and 1b) are synthesized directly from the capped genomic RNA. The replicase gene of the SARS-CoV includes many kinds of polyproteins which are produced as a consequence of the proteolytic processing of ORF1a and 1b [34]. The expression of ORF1b involves -1 ribosomal frameshifting, a process that essentially depends on two elements, known as "slippery" sequence and a complex pseudoknot structure [40]. Two functional proteinase domains, papain-like cysteine proteinase (PL2PRO) and 3C-like cysteine proteinase (3CLPRO), were identified and responsible for the proteolytic processing of