

少もみられた。特に二本鎖 RNA は、一本鎖アンチセンス RNA に比較して高い抗 HIV-1 活性が得られた。さらに、感染させた健常人の木梢血リンパ球 (PBMCs) においても、*env* の二本鎖 RNA は 14 日間にわたり一本鎖アンチセンス RNA より高く HIV-1 の発現を抑制した。

このように、長鎖二本鎖 RNA は一本鎖アンチセンス RNA より効果的な抗 HIV-1 活性を示したが、哺乳動物細胞において二本鎖 RNA の導入によりインターフェロン反応が誘導され、RNAi の特異的な効果が減少されるという欠点がみられた。

そこで、30 塩基以下の短い二本鎖 RNA を作製した。合成長鎖二本鎖 RNA のうち、もっとも強い抗 HIV-1 活性がみられた *env* の中央部を標的として、3'オーバーハングがある 22 塩基の短い二本鎖 RNA を T7 プロモータを用いて合成し、その抗ウイルス効果を検討した。COS 細胞において *env*-siRNAs は 90 % 以上の抗 HIV-1 活性を示し、特に gp120 の V3 ループと CD4 binding site を標的とした siRNAs からはもっとも高い抗 HIV-1 活性がみられた。また、短い二本鎖 RNA は短い一本鎖 RNA より 2 倍から 13 倍も強く HIV-1 発現を抑制した。さらに、そのときの細胞内における標的遺伝子の mRNA を RT-PCR 法によって検出したところ、83 % 以上の *env* の mRNA 量の減少がみられた。対照群としての siRNAs-ran では抗 HIV-1 活性がほとんどみられなかったことから、siRNAs による特異性が高い RNAi 効果が示された。

これらの結果から、われわれは HIV-1 に対する二本鎖 RNA によって一本鎖 RNA より効果的に HIV-1 の発現を阻害することを見い出した。HIV-1 の構造遺伝子である *gag* および *env* を標的とした二本鎖 RNA によるウイルス発現制御は、*env* の中央部を標的したものでもっとも高い効果が得られた。また、短い二本鎖 RNA (siRNAs) を用いる

ことで、さらに高い特異性で HIV-1 の増殖が抑制できることが示唆された。

以上のことから、400 ~ 500 塩基の二本鎖 RNA はエンドヌクレアーゼで短く切断され、標的 RNA と作用する場合において多くの領域をカバーするため高い抗ウイルス効果が期待できる。ただし、siRNA の場合はピンポイントで標的 RNA に作用するため、そのデザインには

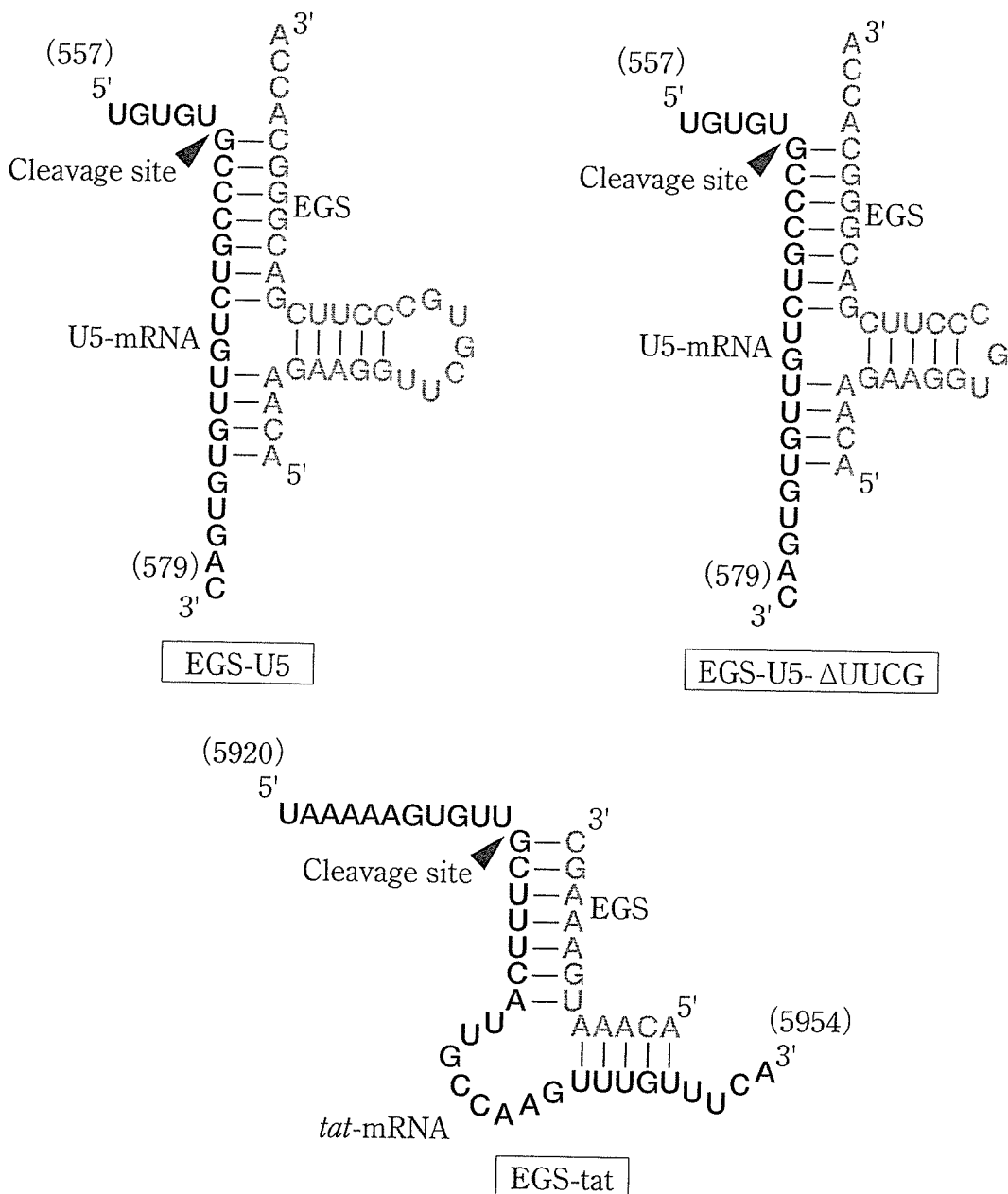


図1 EGS (灰字部分) と標的配列 (黒字部分)

注意が必要であると考えられる。

6. RNase P によるウイルス遺伝子の発現制御²⁷⁾

RNase P で切断する標的遺伝子として HIV-1 の 5'末端側の LTR (long terminal repeat) 領域ならびに遺伝子発現調節因子の一部をコードする *tat* 遺伝子領域を設定した。初めにこれらの領域に特異的に結合する EGSs を設計し、それを哺乳動物細胞内で発現可能なプラスミドベクターに組み込み動物細胞内に導入した (図 1)。EGSs は核内に留まらないように tRNA プロモータまたは U6 プロモータを利用して動物細胞内で EGS-RNA を発現させ、それが切断標的であるウイルス RNA と結合し、tRNA 様の構造をとることで細胞内の RNase P に認識される。それによりウイルス RNA が切断され、ウイルスたんぱく質

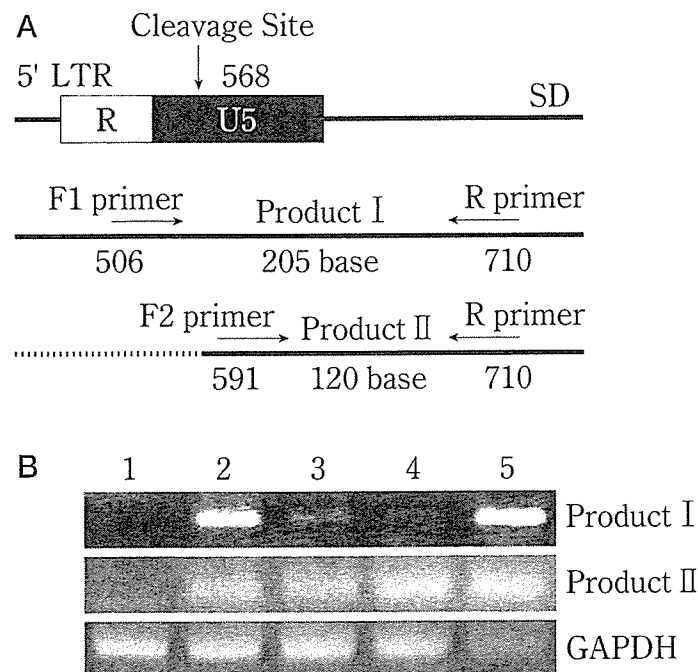


図2 RNase P による標的 RNA の切断を RT-PCR 法にて標的 RNA を検出した結果

A : RT-PCT のプライマー設定部位。Product I ; 全長 RNA を検出するプライマー、Product II ; 切断部位下流の RNA を検出するプライマー。

B : 標的 RNA の検出結果。レーン 1 ; EGS 非導入、レーン 2 ; コントロールベクター導入、レーン 3 ; EGS-U5-DUUCG 導入、レーン 4 ; EGS-U5 導入、レーン 5 ; EGS-*tat* 導入

の発現を制御することが可能になるものと考えられた。

そこで、哺乳動物細胞 (COS 細胞) に EGSs 発現プラスミドベクターと野生型のウイルス遺伝子を導入し、それぞれのベクターから発現した EGS-RNA が HIV-1RNA と結合し、RNase P によって標的 HIV-1RNA が切断されることを RT-PCR 法にて確認した。図 2 に示すように、EGSs 発現ベクターを導入した場合は標的 HIV-1 の RNA 全長が検出されず、切断後の短縮された RNA のみ検出されたことから、EGSs と標的 HIV-1 の RNA 結合による tRNA 様構造を RNase P が認識し、標的 HIV-1 の RNA が切断された。また、このときの培養上清中におけるウイルスたんぱく質量を測定したところ、その産生を 90 % 以上抑制していることが確認された (図 3)。

ここで非常に興味深いことは、RNase P が認識する EGSs と、標的 RNA が形成する tRNA 様構造には、多様性があるという点である。図 1 に示したように、本研究で用いた EGSs は多様な構造を形成するにもかかわらず、標的 RNA の切断ならびにウイルスたんぱく質の発現

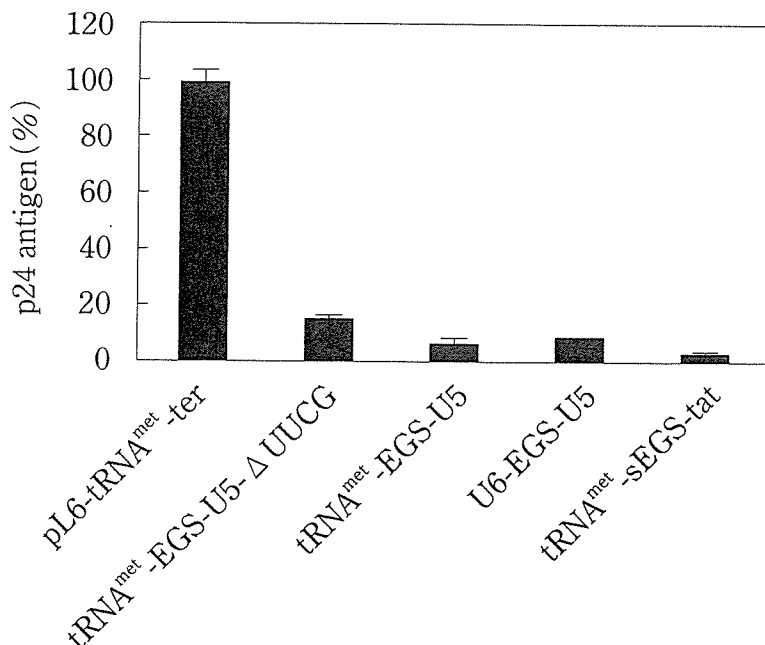


図3 ウイルスたんぱく質発現量

左端のコントロールベクター導入サンプルと比較して EGS 導入細胞ではウイルスの発現をどれも強く抑制した

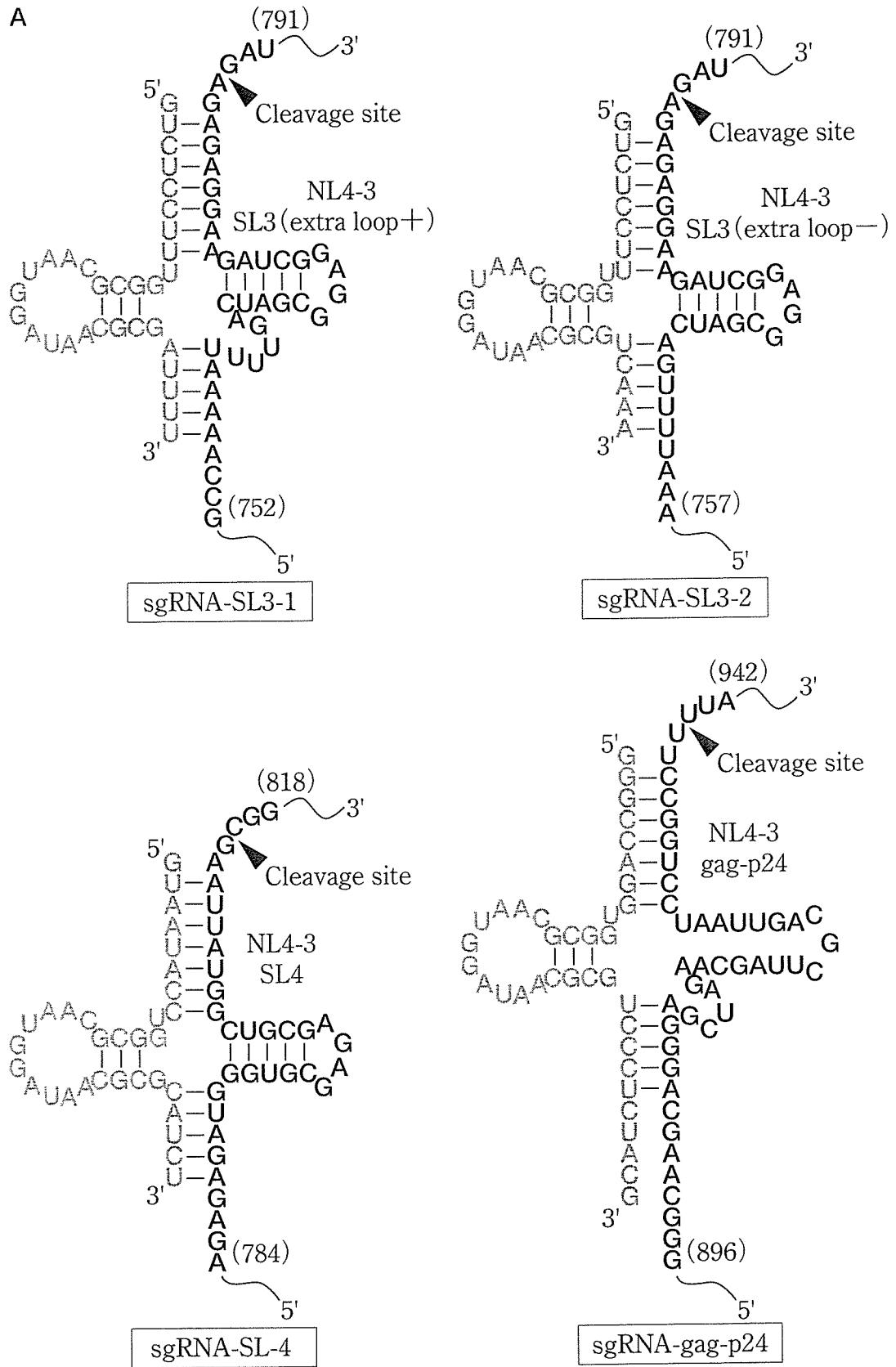


図 4

A : sgRNA とその標的遺伝子 (図 4B、C は次ページに続く)

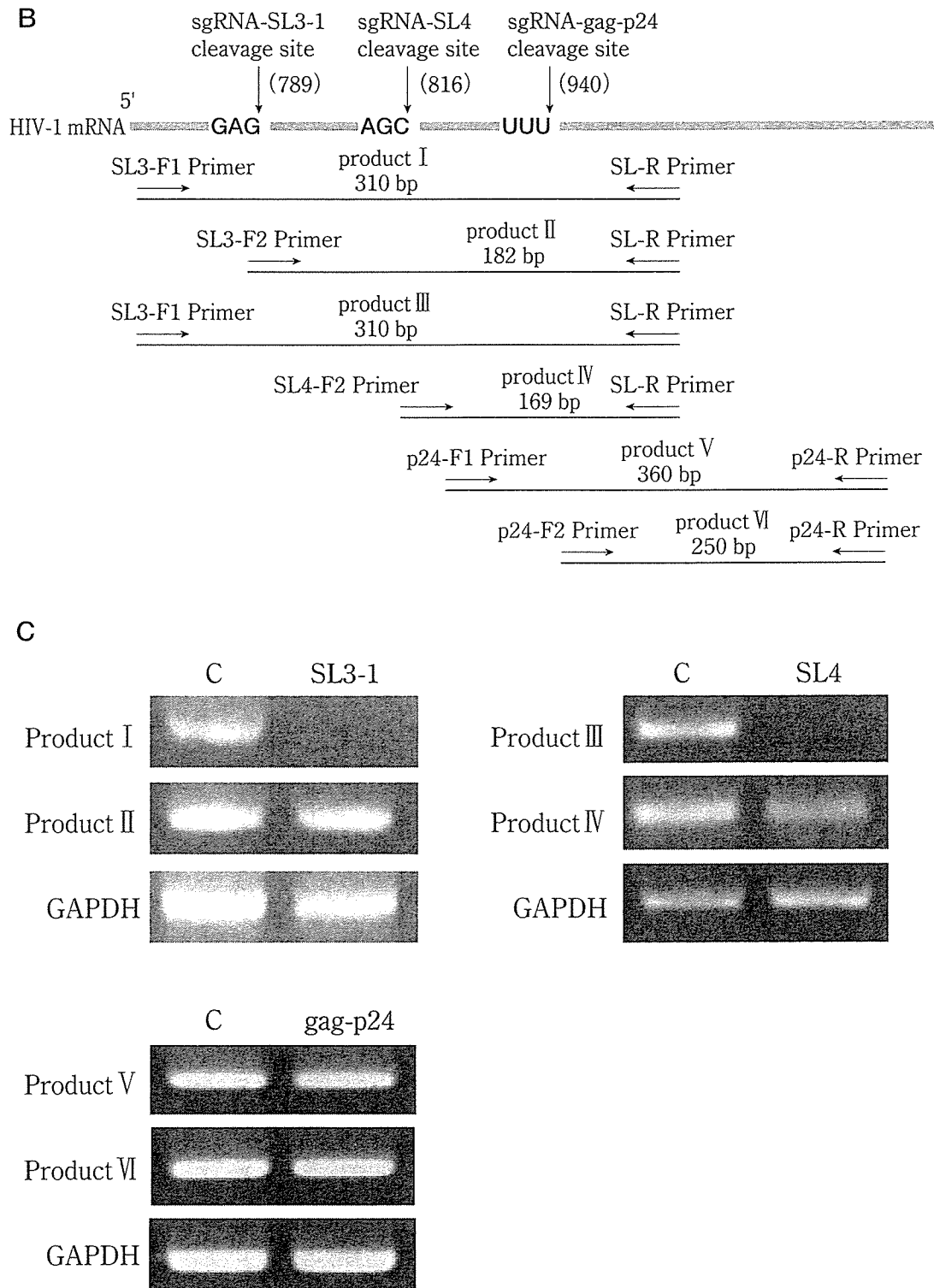


図 4

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 (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ー遺伝子を組み込んだ pNL-luc ベクターとともに細胞

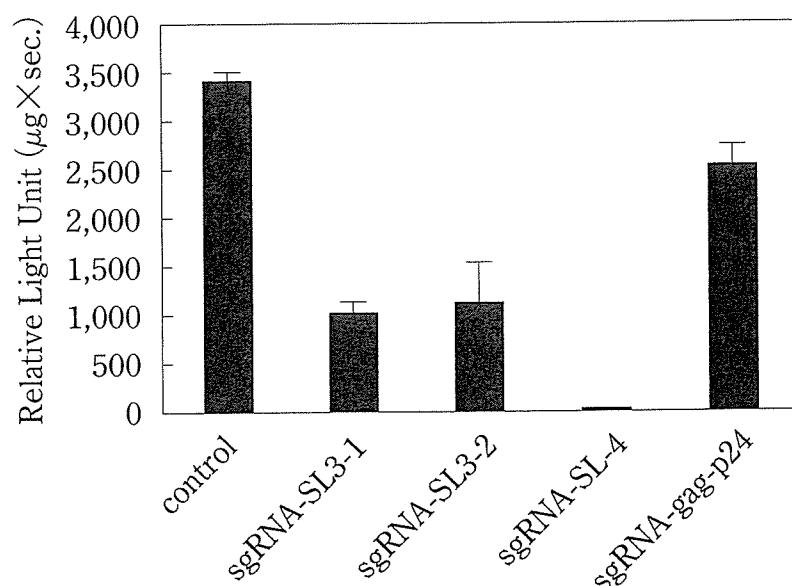


図5 ウイルス由来たんぱく質の発現量を定量

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) は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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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,

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- κ B 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.

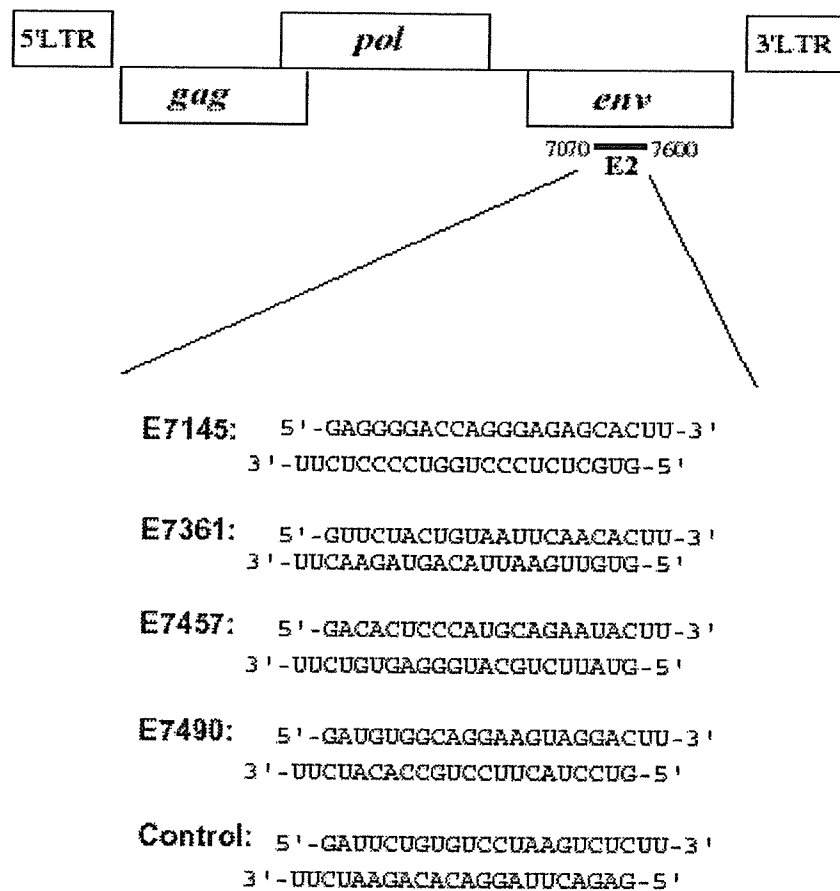


Figure 1. The target sites and the sequences of the siRNAs used in this study. The sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting the HIV-1_{NL4-3} *env* gene are shown. The siRNA duplexes are composed of 22-nucleotides (nt) with 2-nt ribo-uridine 3' overhangs, and are numbered 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 GN₁₈CN₂ 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 SilencerTM siRNA Labeling kit (Ambion, Texas, USA), with several kinds of transfection reagents (LipofectamineTM 2000^{12,18}, Lipofectin, and Oligofectamine) by the FACS Calibur and CellQuest software, revealed that the FITC-labeled-siRNAs encapsulated with LipofectamineTM 2000 strongly associated with the target cells (data not shown). Co-transfection of the plasmid (pNL4-3) and siRNAs was carried out using LipofectamineTM 2000. The virus production in the culture supernatant was assessed by an HIV-1 p24 CLEIA assay²⁰ 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

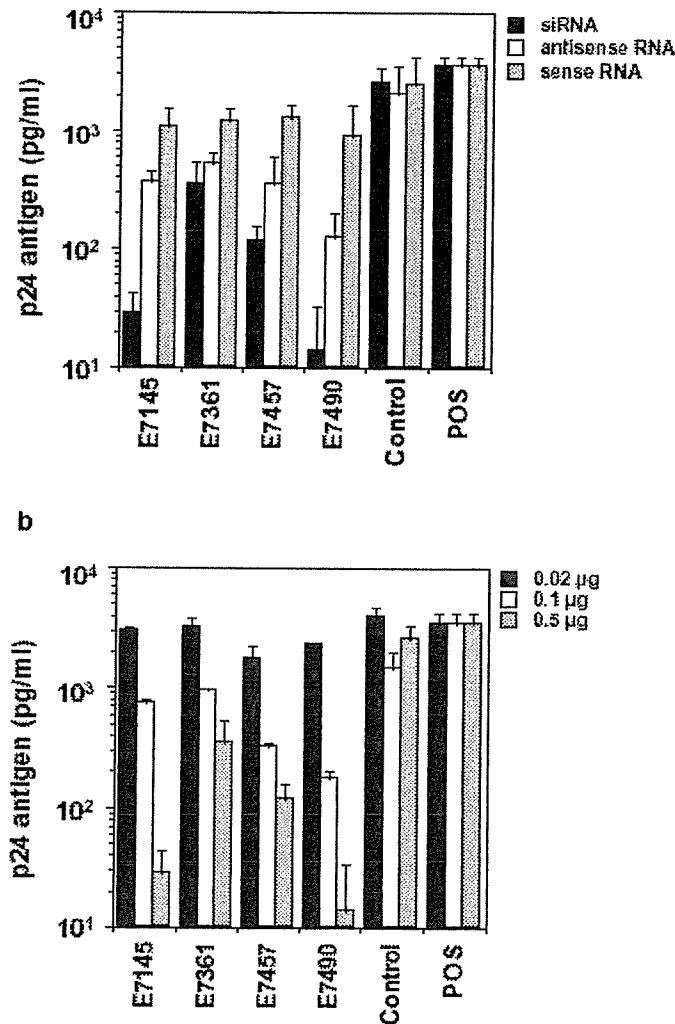


Figure 2. Specific inhibition effects by siRNAs directed against HIV-1 *env*. (a) Inhibitory effects of siRNA duplexes, sense and antisense RNAs on HIV-1 gene expression. Twenty-four h before transfection, COS cells were seeded in 35-mm dishes at a density of 10^5 cells. Transient transfection of siRNAs was carried out using LipofectamineTM 2000 (Invitrogen, Groningen, Netherlands). A 48 μ l aliquot of RPMI-1640 medium without serum was mixed with 2 μ l of LipofectamineTM 2000 per dish, and the mixture was preincubated for 5 min at room temperature. During this incubation, another 50 μ l aliquot of RPMI-1640 medium was mixed with pNL4-3 (0.1 μ g) and the various single-stranded or double-stranded siRNAs (0.5 μ g) 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 μ g) and various amounts of siRNAs (0.02-0.5 μ g), using LipofectamineTM 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 LipofectamineTM 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 LipofectamineTM 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

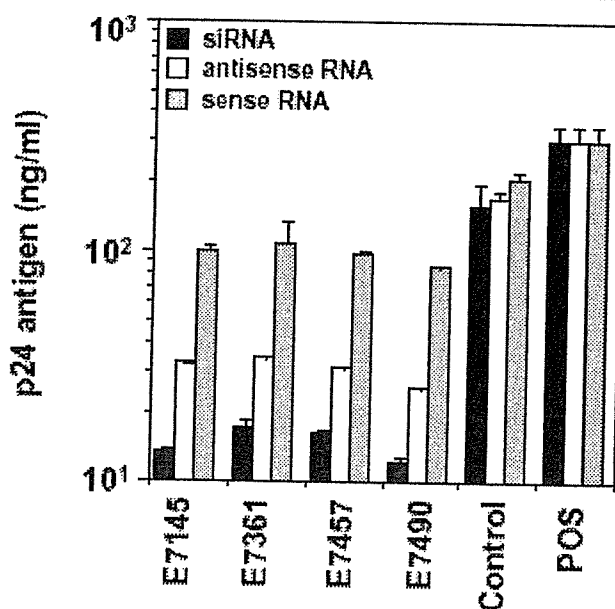


Figure 3. siRNA-mediated inhibition of HIV-1 replication in HIV-1 infected HeLa-CD4⁺ cells. HeLa-CD4⁺ cells (2×10^5 cells) were seeded into 6-multiwell plates 24 h before transfection, and then were transfected with the various single-stranded or double-stranded siRNAs (0.5 μ g), using LipofectamineTM 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 assayed 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.

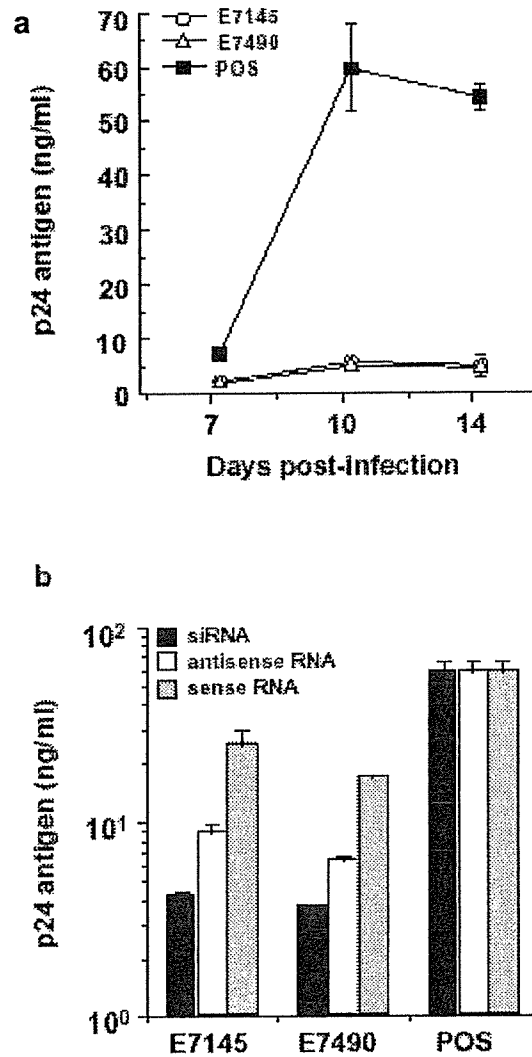


Figure 4. Effects of siRNAs directed against HIV-1 *env* in HIV-1 infected PBMCs. (a) Time course of siRNA-mediated RNAi. Human peripheral blood mononuclear cells (PBMCs) from healthy HIV-1 negative donors were isolated by Ficoll-Hypaque gradient centrifugation, grown in RPMI-1640 medium supplemented with 10% FBS, and activated with 1 μ g/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 μ g). 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 μ g), using LipofectamineTM 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 μ g) encapsulated with LipofectamineTM 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- κ B 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-shRNAs 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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