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Original article

A novel role for Vpr of human immunodeficiency virus type 1 as a regulator of the splicing of cellular pre-mRNA

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Abstract

Vpr, one of the accessory gene products of human immunodeficiency virus type 1 (HIV-1), affects aspects of both viral and cellular proliferation, being involved in long terminal repeat (LTR) activation, arrest of the cell cycle at the G2 phase, and apoptosis. We have discovered a novel role for Vpr as a regulator of the splicing of pre-mRNA both in vivo and in vitro. We found, by RT-PCR and RNase protection analysis, that Vpr caused the accumulation of incompletely spliced forms of *α-globin 2* and *β-globin* pre-mRNAs in cells that had been transiently transfected with a Vpr expression vector. We postulated that this novel effect of Vpr might occur via a pathway that is distinct from arrest of the cell cycle at G2. By analyzing splicing reactions in vitro, we showed that Vpr inhibited the splicing of *β-globin* pre-mRNA in vitro. The splicing of intron 1 of *α-globin 2* pre-mRNA was modestly inhibited by Vpr but the splicing of intron 2 was unaffected. Interestingly, an experimental infection system which utilizes high-titered HIV-1/vesticular stomatitis virus G protein showed that Vpr expressed from an HIV-1 provirus was sufficient to accumulate endogenous *α-globin 2* pre-mRNA. Thus, it is likely that Vpr contributes to selective inhibition of the splicing of cellular pre-mRNA.

Keywords: Human immunodeficiency virus type 1; Vpr; Splicing; Pre-mRNA

1. Introduction

The genome of human immunodeficiency virus type 1 (HIV-1) contains both structural genes, such as *gag*, *pol*, and *env*, and accessory genes, such as *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef*. The *vpr* gene encodes a protein of 96 amino acids that is incorporated in significant quantities into virions [1,2]. Vpr is a nucleophilic protein with non-classical nuclear localization signals [3,4]. The presence of Vpr in the viral particle facilitates efficient infection of macrophages and other non-dividing cells [5–7] by mediating the active nuclear import of preintegration complexes (PIC) [8,9]. One of the important functions of Vpr is the promotion of growth arrest at the G2/M phase of the cell cycle [10–13]. Indeed, there are sufficient amounts of Vpr in incoming viral particles to induce G2 arrest of the cell cycle even prior to the initiation of the synthesis of viral proteins de novo [14,15]. In addition, G2 arrest enhances viral replication, in part by increasing the activity of the long terminal repeat (LTR) [16]. Other evidence also suggests that Vpr can regulate apoptosis both positively and negatively [17–22]. Vpr has also been reported to produce herniation and disruption of the nuclear envelope, which might be correlated with G2 arrest and suggests the possibility that Vpr might allow PICs to bypass the size restrictions of nuclear pore complexes [23].

Splicing of pre-mRNA is not only a nearly ubiquitous and essential step in gene expression but it is also an important mechanism for the generation of protein diversity and the regulation of gene expression. The splicing reaction is performed by the spliceosome, which consists of five small nuclear ribonucleoprotein (snRNP) complexes, namely, U1, U2, U4, U5, and U6, and a large number of non-snRNPs, which include members of the serine- and arginine-rich (SR)

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family of proteins. The spliceosome acts, through a multitude of RNA–RNA, RNA–protein and protein–protein interactions, to excise each intron precisely and to join exons in the correct order [24]. In mammalian cells, SR proteins and other splicing factors are present in regions of the nucleus known splicing factor compartments, nuclear speckles, or interchromatin granules, and they appear to function at several steps in gene expression [25]. Several viral proteins have also been shown to regulate the splicing of pre-mRNAs. For example, NS1 of influenza virus [26,27] and ICP27 of herpes simplex virus (HSV) [28–31] were reported to interfere with splicing of cellular pre-mRNA as part of the mechanism for blockage of host protein synthesis. Moreover, it has been proposed that production of unspliced or partially spliced transcripts of HIV-1 and other lentiviruses might be mediated by the action of virus-encoded Rev, which binds to a specific RNA sequence (the Rev-responsive element or RRE). Rev binds viral RNAs that contain an RRE [32,33] and it exports HIV-1 RNA to the cytoplasm in a CRM1-dependent manner [34–36].

In the present study, we discovered a novel role for Vpr of HIV-1. We demonstrated that Vpr inhibits the splicing of cellular pre-mRNA both in vivo and in vitro. In analyses by RT-PCR and RNase protection assays, we showed that Vpr-induced the accumulation of unspliced forms of *α-globin 2* and *β-globin* pre-mRNA. Furthermore, we confirmed that Vpr inhibited pre-mRNA splicing in an in vitro splicing assay using *β-globin* pre-mRNA as the substrate. We also present strong evidence that Vpr contributes to selective inhibition during the splicing of cellular pre-mRNA. In addition, we demonstrated that HIV-1 infection was sufficient to inhibit splicing of *α-globin 2* pre-mRNA using vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1 viruses. Our results reveal a novel function of Vpr and contribute to an enhanced understanding of splicing mechanisms and the life cycle of HIV-1.

2. Materials and methods

2.1. Cells, transfection and extraction of RNA and DNA

Human cervical HeLa cells and human 293T cells were grown in Dulbecco's modified Eagle's medium that contained 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. The Jurkat line of human T-lymphoid cells was grown in similarly supplemented RPMI 1640 medium.

Transfections were performed by electroporation in a 4-mm-diameter cuvette using a Gene Pulser (Bio-Rad, Richmond, CA.) at 300 V and 975 μF for HeLa cells and at 260 V and 975 μF for Jurkat cells.

Genomic DNA was extracted from HeLa cells with a Wizard™ genomic DNA purification kit (Promega, Madison, WI.). Total RNA was extracted from HeLa cells and Jurkat cells using TRIzol™ reagent (Invitrogen, Carlsbad, CA.).

2.2. Construction of plasmids

The derivative of the expression vector pME18neo that encodes Flag-tagged wild-type Vpr, namely, pME18Neo-Fvpr, has been described previously [37,38]. To generate the control vector pME18Neo-Stop, in which a stop codon was inserted at the amino terminus of the *vpr* sequences, we performed PCR using pME18Neo-Fvpr as the template and primers VprSTOP (5′-ATCCGAATAAGCCCCAGAAGACC-3′) and PMER2 (5′-GGGGAGGTGTGGGAGGTTTT-3′). Then we subcloned the mutated *vpr* gene between the *Eco*RV and *Not*I sites of pME18Neo-Fvpr. To generate the expression vector for the *β-globin* gene, we amplified the *β-globin* gene, including three exons and two introns, by PCR with genomic DNA from HeLa cells as template and primers βG-1-EcoRV-5′ (5′-GGCGATATCCATGGTGCACCTGACTCCT-3′) and βG-end-XbaI-3′ (5′-GCTCTAGATTAGTGATACTTGTGGGC-3′). Then we cloned the amplified fragment between the *Eco*RV and *Xba*I sites of pBluescript II (SK$^+$) (Stratagene, La Jolla, CA.). The resulting construct was designated pSK-β-globin. The *Eco*RV and *Xba*I fragment of pSK-β-globin was excised and subcloned into pME18Neo-HA and encoded the following amino acid sequence: M-A-Y-P-Y-D-V-P-D-Y-A-COOH. To generate the expression vector for the *α-globin 2* gene, we amplified the *α-globin 2* gene, including three exons and two introns, by PCR with genomic DNA from HeLa cells as template and primers HBA2EcoRV (5′-ATCCATGGTGCTGTCTCCTGCC-3′) and HBANotI (5′-CAGCGGCCGCTTAACGGTATTTGGAGG-3′). Then we cloned the amplified fragment between the *Eco*RV and *Not*I sites of pME18Neo-HA.

Infectious molecular clone HIV-1 pNF462 was a kind gift from A. Adachi, Tokushima University, Japan [39]. To generate *env*-negative mutant designating pNF462Δ*env*, frame sift was introduced at *env* region of pNF462 as described below. Parental clone pNF462 was digested using *Bst*EII (TOYOBO, Osaka, Japan) and blunt-ended by KOD (TOYOBO). Then the fragment was self-ligated to introduce 5-base frame sift. To generate *vpr*-negative mutant designating pNF462Δ*env*Δ*vpr*, *Nde*I-*Sal*I fragment of pNL432Δ*vpr* were inserted at *Nde*I-*Sal*I site of pNF462Δ*env*. The generation of pNL432Δ*vpr* was described previously [40]. The expression plasmid of VSV-G, designated pCMV-G, has been previously described [41].

2.3. Reverse transcription-PCR

Samples of RNA were treated with RNase-free DNase I (Invitrogen) for 30 min at room temperature to remove genomic and plasmid DNA. Then 4 μg of total RNA were reverse-transcribed in the presence of oligo(dT) by SuperScript™ II Reverse Transcriptase (Invitrogen) in a total volume of 20 μl.

We amplified intron 1 of endogenous *α-globin 2* pre-mRNA by PCR using primers 5′HBA2E1N1 (5′-TTCTGGTCCCCACAGACTCA-3′) and 3′HBA2E3N1 (5′-

TTATTCAAAGACCAGGAAGGGC-3′) for the first PCR (15 cycles; see below for details of cycles), primers and 5′HBA2I1N2 (5′-GACCCACAGGCCACCCTCAA-3′) and 3′HBA2E3N2 (5′-GTGCTCACAGAAGCCAGGAACTTG-3′) for the second nested PCR or 5′HBA2E1N2 (5′-CCCACCATGGTGCTGTCTCC-3′) and 3′HBA2I2 (5′-CAGTGGCTTAGGAGCTGTGCAG-3′) for 35 cycles, as shown in Fig. 1D. We amplified endogenous *α-globin 2* mRNA by PCR (34 cycles) using primers 5′HBA2E1N1 (5′-TTCTGGTCCCCACAGACTCA-3′) and 3′HBA2E3N1 (5′-TTATTCAAAGACCAGGAAGGGC-3′). Introns 1 and 2 of unspliced *α-globin 2* pre-mRNA and spliced *α-globin 2* mRNA were detected by PCR with 0.5 μl of cDNA as template and the primers shown in Fig. 1D. To detect intron 1 of exogenous *α-globin 2* pre-mRNA, we performed PCR for 32 cycles with primers 5′HBA2I1 (5′-GACCCACAGGC-CACCCTCAA-3′) and 3′HBA2E3 (5′-TAACGGTATTT-GGAGGTCAGCACG-3′). To detect intron 2 of exogenous *α-globin 2* pre-mRNA, we performed PCR for 34 cycles with primers 5′HBA2E1 (5′-CGAGTATGGTGCGGAGGC-3′) and 3′HBA2I2. To detect exogenous *α-globin 2* mRNA, we performed PCR for 23 cycles with primers 5′HBA2E1 and 3′HBA2E3. We amplified *β-actin* mRNA by PCR (16 cycles) using primers 5′β-act (5′-CGTCGCCCTGGACTTCGAGCA-3′) and 3′β-act (5′-GCTGGAAGGTGGACAGCG-AGGCCAGGA-3′). The details of each cycles of PCR were as follows: 2 min at 94 °C; then the indicated number of cycles of incubation at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 45 s; with a final 2-min extension at 72 °C. Reaction products were subjected to electrophoresis on a 2% agarose gel.

All products of PCR were cloned into pBluescript II (SK$^+$) and their identities were confirmed by nucleotide sequencing by the dideoxy chain-termination method with a CEQ^{TM} 2000 DNA-analysis system (Beckman-Coulter, Fullerton, CA.).

2.4. Quantitative PCR

The real-time quantitative PCR of exogenous *α-globin 2* pre-mRNA was performed on LightCycler system (Roche Diagnostics, Mannheim, Germany) in the presence of LightCycler-FirstStart DNA Master SYBR Green I (Roche Diagnostics) using the following primers AG/1stF (5′-TTCTGGTCCCCACAGACTCA-3′) and AG/1stR (5′-TTATTCAAAGACCAGGAAGGGC-3′) for the first PCR, and AG/2nd/IN1F (5′-AGGCCACCCTCAACCGT-3′) and AGExon2R (5′-CTTGAAGTTGACCGGGTC-3′) for the second PCR. For normalization, quantitation of total *α-globin 2* RNA was performed using primers AG/1stF and AG/1stR for the first PCR, and AGExon2F (5′-GATGTTCCTGTCC-TTCCC -3′) and AGExon2R for the second PCR. The first PCR product was treated with ExoSAP-IT^{TM} (containing exonuclease I and shrimp alkaline phosphatase) to remove the first PCR primers according to the manufacturer's instructions (Amersham Bioscience, Uppsara, Sweden) and subjected to the second PCR.

2.5. RNase protection assay

RNase protection assays were performed with an RPA III kit (Ambion, Austin, TX.) according to the instructions in the manual from the manufacture. In brief, 10 μg of total RNA were allowed to hybridize with 8×10^4 cpm ^{32}P-labeled probe overnight at 46 °C. Unprotected single strand RNA was digested with RNases A and T1 and protected fragments were fractionated on a 5% polyacrylamide–7 M urea denaturing gel that was then exposed to an imaging plate (Fuji film, Tokyo, Japan).

For the generation of the probe for detection of splicing of *β-globin* pre-mRNA, we amplified full-length intron 1-exon 2 of *β-globin* by PCR using KOD plus DNA polymerase (TOYOBO) with genomic DNA from HeLa cells as template. We cloned the product of PCR into pBluescript II (SK$^+$) at the *Eco*RV site. The construct was verified by nucleotide sequencing. After linearization with *Not*I, an antisense probe was generated using a Riboprobe System (Promega) according to the manufacturer's standard protocol, 50 μCi [^{32}P]CTP (PerkinElmer, Boston, MA.) and T7 RNA polymerase. At the end of the reaction, 20 units of RNase-free DNase I (Promega) were added and incubation was continued at 37 °C for 15 min. The probe was then purified by gel filtration on a Sephadex G-50 spin column (Amersham Biosciences) to remove unincorporated nucleotides.

2.6. In vitro splicing assay

The pSK-β-globin plasmid including three exons and two introns, was linearized with *Bam*HI and then transcribed by the Riboprobe system (Promega) with T7 RNA polymerase and 50 μCi [^{32}P]CTP (PerkinElmer). Splicing reactions were carried out as described previously [42]. In brief, approximately 25 fmol of RNA transcript were incubated for 2 h at 30 °C with 60% (v/v) nuclear extract in Dignam's buffer D with 20 mM creatine phosphate, 3 mM $MgCl_2$, 0.8 mM ATP, and 2.6% (w/v) polyvinyl alcohol. HeLa nuclear extracts were prepared basically as described previously [43]. Transcripts were separated on a 7% polyacrylamide–7 M urea denaturing gel which was exposed to an imaging plate.

2.7. Western blotting

Cells were lyzed with a 1% solution of SDS 24 or 48 h after transfection. Lysates were suspended in Laemmli's [44] buffer and equal amounts of total protein were fractionated by SDS-PAGE (15% polyacrylamide). The separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA.) for analysis by immunoblotting. Each membrane was blocked for 1 h in a 5% (w/v) solution of skim milk powder in PBS prior to incubation with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO.). Then the membrane was incubated with horseradish peroxidase-linked sheep antibodies against mouse IgG (Amersham Biosciences). Bands of immunoreac-

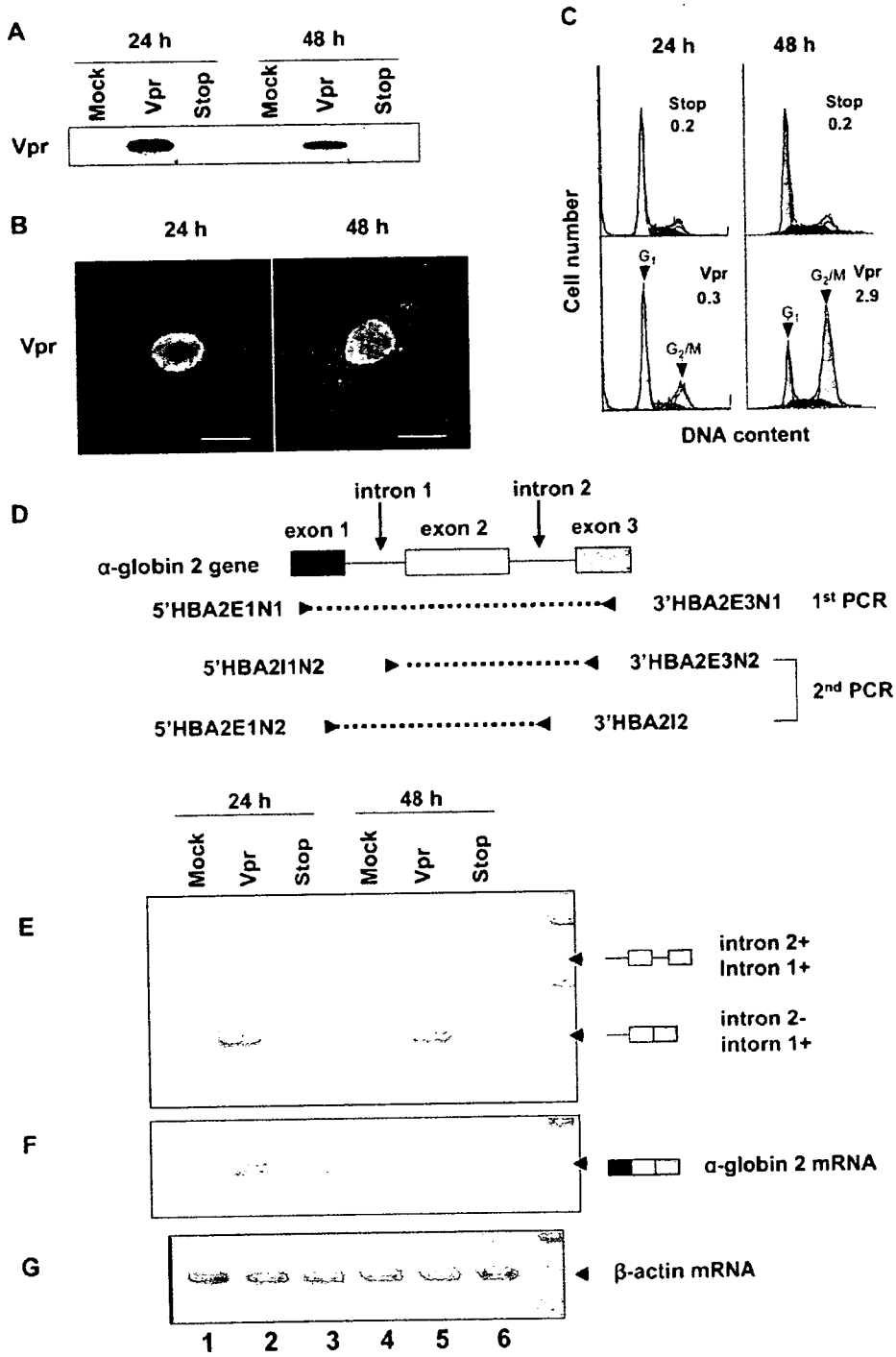

Fig. 1. Vpr prevented splicing of endogenous intron 1 of *α-globin 2* pre-mRNA in HeLa cells. HeLa cells were transfected with 30 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (Vpr) or with 30 μg of control pME18Neo-Stop (Stop), or they were mock-transfected. Then, 24 and 48 h after transfection, cells were subjected to Western blotting (A), immunofluorescence (B), analysis of the cell cycle (C) and RT-PCR (D–G). (A) Western blotting of Vpr. Transfected cells were lyzed and then lysates containing 25 μg of protein were subjected to Western blotting analysis with the Flag-specific MAb M2. (B) Subcellular localization of Vpr. Transfected cells were subjected to immunofluorescence staining with Flag-specific MAb M2 and Alexa 488-conjugated goat antibodies against mouse IgG and analyzed by confocal laser scanning microscopy. Bar, 20 μm. (C) The DNA content of HeLa cells that expressed Vpr. Transfected cells were treated with the Flag-specific monoclonal antibody M2 and propidium iodide (PI). Cells that bound M2 were quantitated by flow cytometry. Arrowheads indicate peaks of cells at the G1 and G2/M phase. The ratio of cells at G2/M to those at G1 is indicated in the upper right of each graph. (D) Schematic representation of the human *α-globin 2* gene and the positions of primers used for nested RT-PCR. (E) Result of nested RT-PCR for examination of endogenous *α-globin 2* pre-mRNA. Total RNA was extracted from cells, as indicated, and subjected to the first RT-PCR with primers specific for exon 1 (5'HBA2E1N1) and

tive proteins were detected with the SuperSignal™ West Pico chemiluminescent substrate (Pierce, Rockford, IL.).

2.8. Immunofluorescence assay

HeLa cells, growing on coverslips, were examined 24 or 48 h after transfection by an immunofluoresence assay, as described previously [4].

2.9. Analysis of the cell cycle

HeLa cells were harvested 24 or 48 h after transfection and analyzed by flow cytometry for DNA content, as described previously [45].

2.10. Virus infection assay

To generate VSV-G pseudotyped virus, 293T cells (1×10^6 cells) were transfected with 5 μg of VSV-G expression vector together with 10 μg of pNF462Δ*env* or pNF462Δ*env*Δ*vpr*. Supernatants of transfected cells were harvested 24 h after transfection, filtered through 0.45-μm-pore-size filters and treated with DNase I (250 U, Sigma). The amount of HIV-1 p24 antigen was quantified with Lumipulse (Fujirebio, Tokyo Japan). HeLa cells were infected with VSV-G pseudotyped virus containing 100 ng of p24 antigen per 5×10^5 cells.

3. Results

3.1. Accumulation of the incompletely spliced form of endogenous α-globin 2 pre-mRNA

Vpr is involved in the import of the PIC of HIV-1 into the nuclei of non-dividing cells, in cellular differentiation, in the induction of cell cycle arrest at the G2/M phase, in immune suppression, and in enhancement of the replication of the HIV-1 itself [10–14,16,46]. However, although Vpr is a nucleophilic protein with non-classical nuclear localization signals [3,4], the function of Vpr in the nucleus remains to be clarified. To examine whether Vpr might affect splicing by the spliceosome, which is an important event in the nucleus, we transfected HeLa cells with pME18Neo-Fvpr that encoded Flag-tagged Vpr and with the control vector pME18Neo-Stop, in which a stop codon was located at the amino terminus of the *vpr* sequence. We extracted total cellular RNA and examined levels of unspliced endogenous human *α-globin 2* pre-mRNA and of spliced mRNA by nested RT-PCR (Fig. 1). We selected the human gene for *α-globin 2* as the cellular target gene, because it is a simple construct with only three exons and two introns and the length of the coding region, including two introns, is only 685 bps. Vpr was expressed at detectable levels within 24 h after transfection (Fig. 1A) and was localized predominantly in the nucleus and nuclear envelope (Fig. 1B). However, it was minimally effective in inducing G2 arrest (Fig. 1C). To our surprise *α-globin 2* pre-mRNA containing intron 1 but not intron 2 was clearly detectable in HeLa cells that had been transiently transfected with the Vpr-coding vector after the second PCR with forward primer 5′HBA2I1N2, which is located in intron 1, and reverse primer 3′HBA2E3N2, which is located in exon 3. We sequenced the fragment obtained by PCR and confirmed that the fragment was *α-globin 2* pre-mRNA that retained intron 1 but lacked intron 2 (data not shown). By contrast, pre-mRNA that contained intron 2 did not accumulate in cells that had been transfected with the Vpr-coding vector, or in cells transfected with the control vector pME18Neo-Stop and in mock-transfected cells. In addition, the second PCR with forward primer 5′HBA2E1N2, which spans exon 1, and reverse primer 3′HBA2I2, which spans intron 2, yielded neither of two possible types of pre-mRNA that contained intron 2 (data not shown). By contrast, in all cells transfected with the control vector pME18Neo-Stop and all mock-transfected cells, essentially all of the *α-globin 2* pre-mRNA had been spliced and spliced mRNA was produced (Figs. 1E, F). The levels of expression of *β-actin* mRNA were the same in all samples examined (Fig. 1G), as were levels of mRNAs that encoded GAPDH and L13a (data not shown). Our results indicated that splicing of *α-globin 2* pre-mRNA had been partially inhibited by overexpression of Vpr, allowing incompletely spliced pre-mRNA to accumulate. Moreover, we obtained similar results later in 48 h period after transfection, namely, during the time when Vpr is able to induced significant G2 arrest, as shown in Fig. 1C. These observations suggest that the Vpr-induced inhibition of splicing and G2 arrest are independent phenomena.

3.2. Vpr inhibits splicing of exogenous β-globin pre-mRNAs

To confirm that Vpr affects splicing of pre-mRNA in vivo, we selected the human gene for *β-globin*, which is also a comparatively simple construct. We produced a derivative of pME18Neo that included the *β-globin* gene, with three exons and two introns, under the control of the SRα promoter, as shown in Fig. 2A. Then, we transiently transfected HeLa cells with two pME18Neo expression vectors, namely, one that encoded Vpr and one that encoded *β-globin* pre-mRNA. We examined the splicing of *β-globin* pre-mRNA by RNase protection analysis using a ^{32}P-labeled antisense probe specific for *β-globin* pre-mRNA (Fig. 2). This probe was able to dif-

exon 3 (3′HBA2E3N1), and then the second nested RT-PCR with primers specific for intron 1 (5′HBA2I1N2) and exon 3 (3′HBA2E3N2), or with specific primers of exon 1 (5′HBA2E1N2) and intron 2 (3′HBA2I2). (F) Result of RT-PCR for examination of *α-globin 2* mRNA with primers specific for exon 1 (5′HBA2E1N1) and exon 3 (3′HBA2E3N1). (G) Result of RT-PCR for examination of *β-actin* mRNA. The products of PCR were subjected to electrophoresis on a 2% agarose gel. The data are representative of five independent experiments that gave similar results.

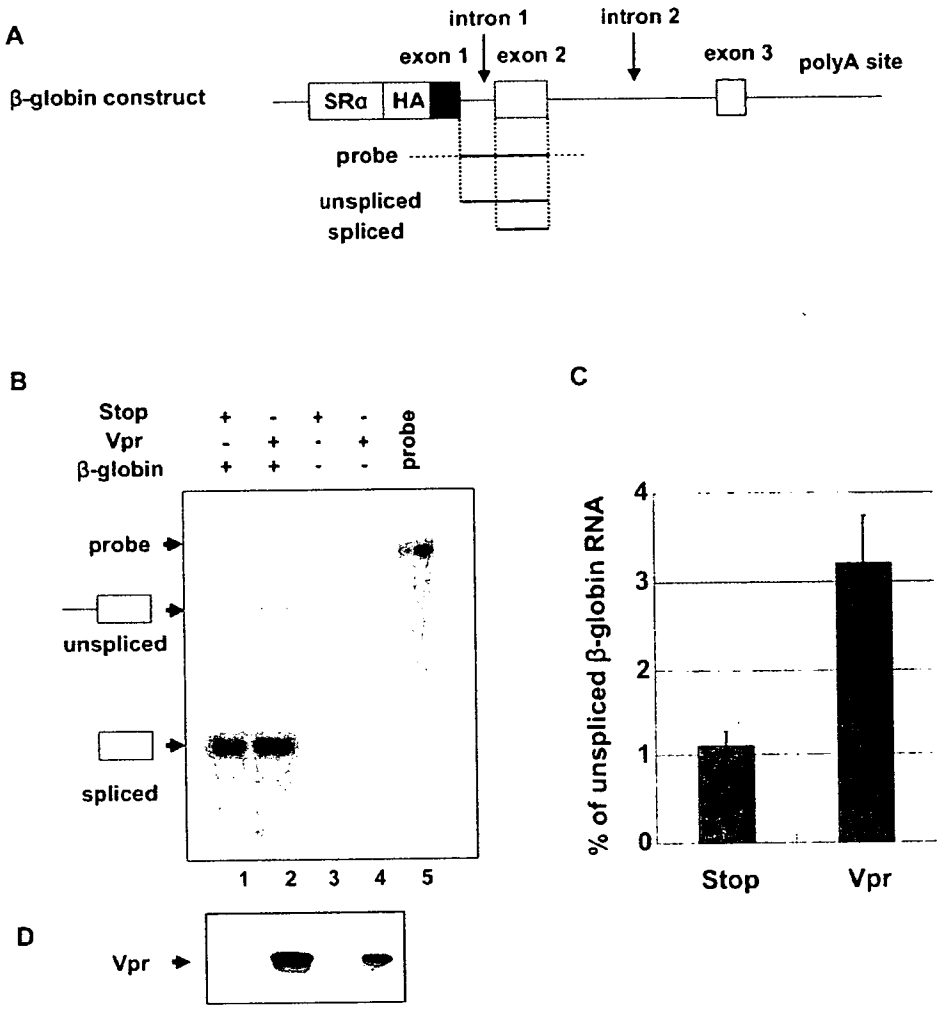

Fig. 2. Vpr inhibited the splicing of exogenous *β-globin* pre-mRNA in HeLa cells that had been transfected with a vector that encoded Vpr and a vector that encoded *β-globin* pre-mRNA. (A) The pME18Neo construct, showing the *β-globin* gene that contained three exons and two introns under the control of the SRα promoter. Bar shows the location of the antisense probe specific for *β-globin* pre-mRNA. In the RNase protection assay, this probe allowed discrimination between unspliced pre-mRNA and spliced mRNA, as indicated. B-D, HeLa cells were cotransfected with 25 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (lanes 2 and 4) or control pME18Neo-Stop (lanes 1 and 3) together 5 μg of pME18Neo that encoded *β-globin* pre-mRNA (lanes 1 and 2). HeLa cells were harvested 24 h after transfection and divided into two portions. (B) Some cells were subjected to an RNase protection assay. Total RNA was extracted from cells and subjected to an RNase protection assay with the ^{32}P-labeled *β-globin* antisense probe. After treatment with RNases A and T1, the protected fragments were separated by electrophoresis on a 5% polyacrylamide–7 M urea denaturing gel. Arrows indicate positions of the intact probe, of unspliced *β-globin* RNA (intron 1+), and of spliced *β-globin* RNA (intron 1-). (C) Intensities of the unspliced and spliced β-globin pre-mRNA signals were quantitated using BAS2500 (Fujifilm Co., Tokyo, Japan) and percentage of the intensity of unspliced *β-globin* RNA against that of unspliced plus spliced *β-globin* RNA was calculated in each transfection. Each column and error bar represent the mean ± S.D. of results from three independent experiments. (D) The remaining cells were subjected to Western blotting with the Flag-specific monoclonal antibody M2 to determine levels of expression of Vpr. The data are representative of three independent experiments that gave similar results.

ferentiate unspliced *β-globin* pre-mRNA that contained intron 1 from spliced mRNA, as shown in Fig. 2A. Protected fragments were fractionated on a 5% acrylamide–7 M urea denaturing gel. We found that, 24 h after transfection, pre-mRNA that contained intron 1 modestly accumulated in cells that had been transfected with pME18Neo-Fvpr plus the *β-globin* expression vector, as compared to levels in HeLa cells that had been transfected with pME18Neo-Stop plus the *β-globin* expression vector (Fig. 2B, lanes 1 and 2). The percentage of unspliced *β-globin* RNA against total *β-globin* RNA in HeLa cells that had been transfected with pME18Neo-Fvpr was approximately threefold higher than that in HeLa cells that had been transfected with control vector pME18Neo-Stop (Fig. 2C). Thus, it appeared that Vpr had modestly inhibited splicing of exogenous *β-globin* pre-mRNA in vivo. Furthermore, *β-globin* pre-mRNA was spliced and spliced *β-globin* mRNA also accumulated. By contrast, no bands of unspliced pre-mRNA and spliced mRNA were detected in the analysis of RNA from HeLa cells had not been transfected with the *β-globin* expression vector (Fig. 2B, lanes 3 and 4), indicating the absence of endogenous *β-globin* pre-mRNA and spliced mRNA in this system. Moreover, we obtained similar

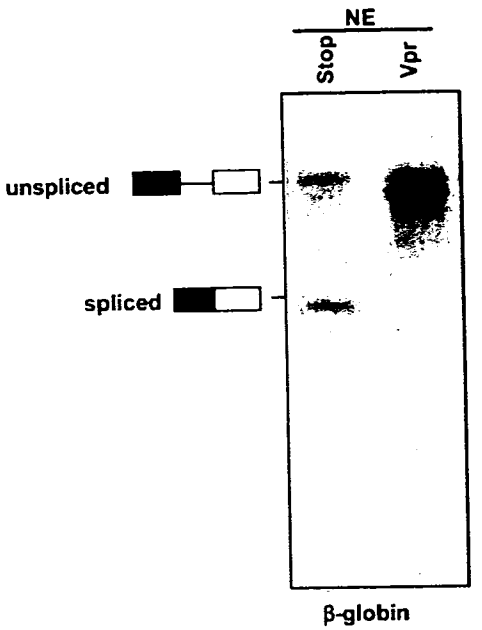

Fig. 3. Vpr inhibited splicing in vitro. HeLa cells were transfected with 30 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (Vpr) or 30 μg of control pME18Neo-Stop (Stop). Then, 24 h after transfection, nuclear extracts (NE) were prepared from cells and subjected to in vitro splicing assay with ^{32}P-labeled *β-globin* pre-mRNA as substrate. The products were separated on a 7% polyacrylamide–7 M urea denaturing gel.

results in RNase protection analysis with a derivative of pME18Neo that included the *α-globin 2* gene with three exons and two introns under the control of the SRα promoter (data not shown). Since our findings tended to confirm the results of amplification by nested RT-PCR of the endogenous pre-mRNA, it appeared that Vpr might partially inhibit the splicing of *β-globin* and *α-globin 2* pre-mRNAs in vivo.

3.3. Vpr inhibits pre-mRNA splicing in vitro

To obtain definitive evidence that Vpr acts on splicing, we included this protein in in vitro splicing assays with a nuclear extract (NE) of HeLa cells that had been transiently transfected with either pME18Neo-Fvpr or pME18Neo-Stop (Fig. 3). As a substrate we used ^{32}P-labeled *β-globin* pre-mRNA with a G capped at the 5′ end. As expected, the splicing of *β-globin* was dramatically suppressed by the nuclear extract from HeLa cells that expressed Vpr. By contrast, splicing activity of a nuclear extract from HeLa cells that had been transfected with pME18Neo-Stop was sufficient to splice *β-globin* pre-mRNA. This in vitro splicing assay demonstrated that Vpr regulated the splicing of *β-globin* pre-mRNA.

3.4. Vpr inhibits splicing of exogenous α-globin 2 pre-mRNA

As shown in Fig. 1, amplification of endogenous *α-globin 2* pre-mRNA by nested RT-PCR demonstrated that pre-mRNA that contained only intron 1 and not intron 2 accumulated in the presence of Vpr. Thus, inhibition of splicing upon expression of Vpr might be sequence-specific. Therefore, we examined the effects of the expression of Vpr on cellular splicing by RT-PCR using total RNA from Jurkat cells that had been transfected with pME18Neo-Fvpr or pME18Neo-Stop and a derivative of pME18Neo that included the *α-globin 2* gene (Fig. 4). In this experiment, we used Jurkat cells, a line of human T-lymphoid cells that is permissive with respect to infection by HIV-1 and from which it is not possible to amplify the endogenous *α-globin 2* pre-mRNA by nested RT-PCR. In our analysis, 24 h after transfection, we detected three products amplification by RT-PCR of pre-mRNA. They contained intron 1 but not intron 2 (Fig. 4Bb), intron1 and intron 2 (Fig. 4Ba, Bc), and intron 2 but not intron 1 (Fig. 4Bd) both in the absence and in the presence of Vpr. The levels of *β-actin* mRNA were similar in all samples examined (Fig. 4B). These results indicate that splicing of intron 1 might be partially inhibited by Vpr, while splicing of intron 2 was unaffected. We obtained similar results in HeLa cells (data not shown). Collectively, our results indicate that Vpr of HIV-1 is involved in selective inhibition during the splicing of cellular pre-mRNAs.

3.5. Vpr expressed from an HIV-1 provirus accumulates endogenous α-globin 2 pre-mRNA

To monitor the potential of Vpr to inhibit the splicing of cellular pre-mRNAs, we assessed whether HIV-1 infection accumulated endogenous *α-globin 2* pre-mRNA. HeLa cells were infected with 100 ng equivalent of p24 of VSV-G pseudotyped HIV-1 *vpr*$^+$ or *vpr*$^-$ virus. Then 24 h after infection, total RNA was isolated from HeLa cells and real-time quantitative RT-PCR were carried out to quantitate the level of endogenous *α-globin 2* pre-mRNA. The level of total *α-globin 2* RNA was measured for normalization and fold-production of the level of *α-globin 2* pre-mRNA that contains intron 1 was quantified (Fig. 5B). Interestingly, a higher level of *α-globin 2* pre-mRNA that contains intron 1 was detected in HeLa cells infected with *vpr*$^+$ virus than that in HeLa cells infected with *vpr*$^-$ virus. This result indicated that Vpr produced from an HIV-1 provirus was sufficient to accumulate endogenous *α-globin 2* pre-mRNA in an infection system as well as in a transient transfection system.

4. Discussion

Our present results lead to three major conclusions. First, the present study reveals that Vpr, when expressed transiently in cells, can regulate the splicing reaction of cellular pre-mRNA both in vivo and in vitro. Using RT-PCR and RNase protection assays, we showed that Vpr induced the accumulation of unspliced forms of *α-globin 2* and *β-globin* pre-mRNAs in HeLa cells that expressed Vpr. We confirmed the modulation of splicing by Vpr in in vitro splicing assays

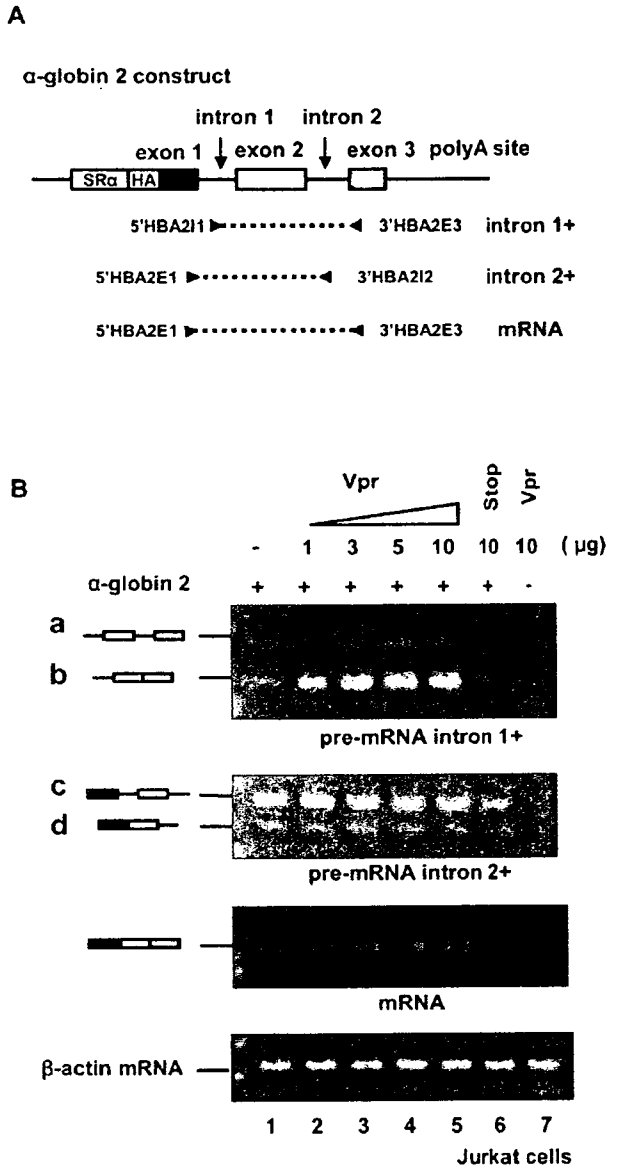

Fig. 4. Analysis by RT-PCR of exogenous *α-globin 2* pre-mRNA in Jurkat cells that had been transfected with a vector that encoded Vpr and a vector that encoded *α-globin 2* pre-mRNA. (A) The pME18Neo construct including *α-globin 2* gene that contained three exons and two introns under the control of the SRα promoter and the position of each primer used for RT-PCR. (B) Jurkat cells were transfected with 1, 3, 5 and 10 μg of pME18Neo that encoded Flag-tagged wild-type Vpr (lanes 2–5), 10 μg of control pME18Neo-Stop (lane 6) or none of this plasmid (lane 1), and 1 μg of pME18Neo that encoded *α-globin 2* pre-mRNA (lanes 1 and 2). Then, 24 h after transfection, RT-PCR was performed with a pair of primers specific for *α-globin 2* pre-mRNA, and products of PCR were subjected to electrophoresis on a 2% agarose gel. RT-PCR to amplify cellular *β-actin* mRNA was performed as a control. Total amounts of DNA were equalized by addition of control pME18neo. The data are representative of results of three independent experiments.

as follows. The splicing of *β-globin* pre-mRNA was dramatically suppressed when a nuclear extract from HeLa cells that expressed Vpr was added to an in vitro splicing system, sug-

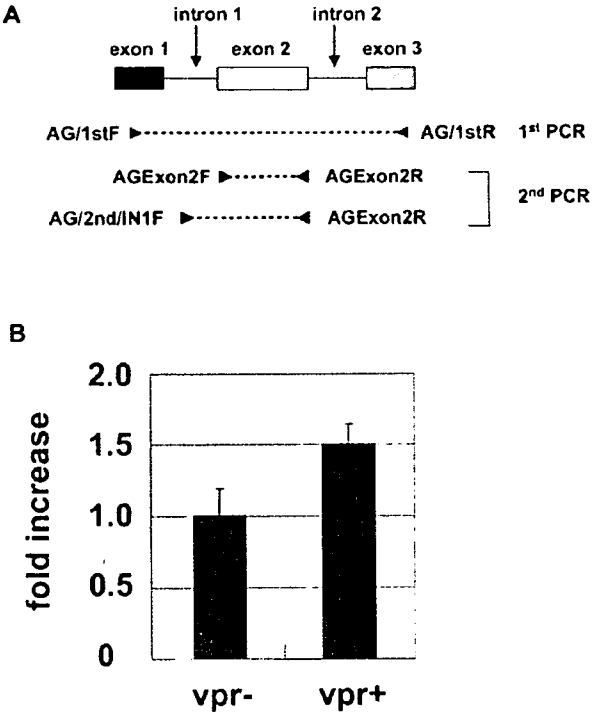

Fig. 5. Vpr expressed from an HIV-1 provirus accumulates endogenous α-globin2 pre-mRNA. Vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1 viruses were harvested from 293T cell cultures 24 h after cotransfection with VSV-G expression vector together with pNF462Δ*env* or pNF462Δ*env*Δ*vpr*. HeLa cells (5×10^5) were infected with 100 ng of p24 antigen equivalent of pseudotyped viruses. Two hours after infection, cells were washed with serum-free medium twice and cultured with fresh medium containing 10% fetal bovine serum. Then, 24 h after infection, quantitative RT-PCR was performed using specific primers for total *α-globin 2* RNA or *α-globin 2* pre-mRNA that contains intron 1. (A) Schematic representation of *α-globin 2* gene and the position of primers used for quantitative RT-PCR. (B) Fold increase of the level of *α-globin 2* pre-mRNA that contains intron 1. Signals of amplification products were normalized by those of total *α-globin 2* RNA. Each column and error bar represent the mean ± S.D. of results from three independent experiments.

gesting that Vpr had an effect on the splicing machinery. The level of inhibition of splicing by Vpr was modest in vivo but high in vitro. The reason why such difference arose between in vivo and in vitro was not clear, but there was a possibility that some factors that could alleviate the inhibition of splicing by Vpr in vivo existed at low levels or lacked in HeLa nuclear extract that used in the in vitro splicing assay. Thus, the mechanism by which Vpr inhibits pre-mRNA splicing appears to be novel. Second, although the inhibition of splicing induced by Vpr was not as strong as that shown in a transient transfection system, we also indicated strong evidence that Vpr expressed from an HIV-1 provirus was sufficient to accumulate endogenous α-globin 2 pre-mRNA in an infection system using VSV-G pseudotyped HIV-1 vpr^+ or vpr^- virus. Third, our results also indicate the potential sequence-specific nature of the Vpr-induced modulation of splicing. Amplification of endogenous *α-globin 2* pre-mRNA by nested RT-PCR demonstrated that pre-mRNA that contained only intron 1 and not intron 2 accumulated in presence of Vpr.

Moreover, RT-PCR with total RNA from Jurkat cells that had been transfected with a pME18Neo-Fvpr plus a derivative of a pME18Neo that included the *α-globin 2* gene revealed that Vpr-induced an increase in the accumulation of all products of pre-mRNA that contained intron 1. Further studies are required to clarify why Vpr inhibit splicing of the particular intron.

The NS1 protein of influenza virus [26,27,47] and the ICP27 protein of HSV [29–31,48] have been reported to inhibit the splicing of cellular pre-mRNA, perhaps as a part of the mechanism for shutting down the synthesis of host proteins. The NS1 protein binds to U6 small nuclear RNA, inhibiting the formation of U4/U6 and U2/U6 complex [47], while ICP27 inhibits splicing by interfering with assembly of spliceosomes [31]. In addition, it has been proposed that ICP27 interacts with SRPK1 and inhibits splicing by altering the phosphorylation of SR protein [48]. Moreover, ICP27 also interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step [30]. It has been demonstrated that Vpr binds to ribonucleic acid via a process that requires the carboxy-terminal basic domain of the protein (in particular the helical 70–80 domain) [49], which suggests the possibility of a functional association with pre-mRNA. Indeed, we have preliminary evidence (Kuramitsu and Aida, personal communication) that a carboxy-terminal domain of Vpr is essential for the inhibition of splicing. Therefore, our present results and the previous demonstration that NS1 and ICP27 can associate with spliceosomes that contained splicing intermediates suggest that Vpr might interact with spliceosomes, which inhibit splicing at the stage when the splicing complex is formed. It is also possible that Vpr inhibits splicing by preventing pre-mRNA from association with spliceosomes or stabilizing pre-mRNA through the association with pre-mRNA. These issues will be further elucidated.

The correlations between the novel ability of Vpr to inhibit splicing of cellular pre-mRNA and the previously characterized functions of Vpr in phenomena such as LTR activation [16], apoptosis [50] and G2 arrest [10–13], suggest some intriguing possibilities. Splicing and transcription are tightly coupled [51]. For example, spliceosomal UsnRNP forms a complex with elongation factor TAT-SF1, which associates with RNA polymerase II (pol II) via the carboxy-terminal domain (CTD) kinase PTEFb. This UsnRNP-TAT-SF1 complex stimulates both transcription and splicing in vitro [52]. Kino and Pavlakis [53] have reported that hsRBP7, a subunit of pol II, bind Vpr in a yeast two-hybrid screening assay. This observation strongly suggests that Vpr might regulate transcription via an interaction with hsRBP7 and might then coincidentally, participate in the splicing reaction. Moreover, apoptosis and splicing influence one another. For example, the cellular apoptosis-promoting factor TIA-1 is a regulator of the splicing of pre-mRNA [54,55]. However, it remains unclear whether Vpr can inhibit splicing via these two processes. By contrast, Vpr-induced G2 arrest might be a key event related to the inhibition of splicing by Vpr. Roshal et al. [56] showed that treatment with LY294002, an inhibitor of phosphatidylinositol 3-kinase, alleviated Vpr-induced G2 arrest in HeLa cells. Moreover, recent reports indicated that RNA processing is also the target of several signal-transduction pathways, including phosphatidylinositol 3-kinase pathway [57,58]. However, in the present study, we detected the accumulation of pre-mRNA during the 24-h period after transfection, namely, during the time when Vpr is unable to induce G2 arrest. This result clearly indicates that the inhibition of splicing by Vpr is not the result of G2 arrest. Further studies are required to determine whether inhibition of the splicing of pre-mRNA influences the induction of G2 arrest by Vpr.

The generation of the 2 or 4-kb form of HIV-1 RNA from unspliced 9-kb genomic RNA by splicing is orchestrated by *cis* elements like exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing silencers, and the viral protein Rev [59–64]. It has been suggested that, Rev-mediated nuclear export of incompletely spliced HIV-1 RNA regulates splicing of HIV-1 RNA [63]. However, splicing of HIV-1 RNA is also controlled by many cellular splicing factors, such as SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). For example, the generation of tat mRNA is regulated via splicing acceptor site A3. Splicing at the A3 site is regulated by ESS2p [62], ESE2 [60] and ESS2 [61]. The ribonucleoprotein designated hnRNP H binds to the ESS2p element to repress activity at splice site A3. SC35 binds to the ESE2 to activate splicing, whereas hnRNP A1 binds ESS2 to repress splicing. ESE2 and ESS2 overlap and binding of hnRNP A1 to ESS2 masks binding site for SC35 and inhibits splicing at the A3 site. By contrast, it has been suggested that Vpr acts multifunctionally via interactions with numerous cellular partner molecules, such as the 14-3-3, the p300/CREB-binding protein, and the importin α [53]. Therefore, it is possible that Vpr might be associated not only with host splicing but also with the alternative splicing of HIV-1 RNA. However, we do not know whether Vpr interacts with regulators of splicing that control splicing of HIV-1 RNA. Thus, while our present results suggest that Vpr inhibits splicing of cellular pre-mRNA, it remains to be determined whether Vpr regulates splicing of the HIV-1 genome. Our understanding of the life cycle of HIV-1 and the progression of AIDS pathogenesis will be enhanced as we improve our understanding of the roles of Vpr both in cellular splicing and in the life cycle of HIV-1.

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References

[1] X. Yuan, Z. Matsuda, M. Matsuda, M. Essex, T.H. Lee, Human immunodeficiency virus vpr gene encodes a virion-associated protein, AIDS Res. Hum. Retroviruses 6 (1990) 1265–1271.

[2] E.A. Cohen, G. Dehni, J.G. Sodroski, W.A. Haseltine, Human immunodeficiency virus vpr product is a virion-associated regulatory protein, J. Virol. 64 (1990) 3097–3099.

[3] Y. Jenkins, M. McEntee, K. Weis, W.C. Greene, Characterization of HIV-1 vpr nuclear import: analysis of signals and pathways, J. Cell Biol. 143 (1998) 875–885.

[4] M. Kamata, Y. Aida, Two putative alpha-helical domains of human immunodeficiency virus type 1 Vpr mediate nuclear localization by at least two mechanisms, J. Virol. 74 (2000) 7179–7186.

[5] N.K. Heinzinger, M.I. Bukinsky, S.A. Haggerty, A.M. Ragland, V. Kewalramani, M.A. Lee, H.E. Gendelman, L. Ratner, M. Stevenson, M. Emerman, The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells, Proc. Natl. Acad. Sci. USA 91 (1994) 7311–7315.

[6] R.I. Connor, B.K. Chen, S. Choe, N.R. Landau, Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes, Virology 206 (1995) 935–944.

[7] P. Gallay, V. Stitt, C. Mundy, M. Oettinger, D. Trono, Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import, J. Virol. 70 (1996) 1027–1032.

[8] R.A. Fouchier, B.E. Meyer, J.H. Simon, U. Fischer, A.V. Albright, F. Gonzalez-Scarano, M.H. Malim, Interaction of the human immunodeficiency virus type 1 Vpr protein with the nuclear pore complex, J. Virol. 72 (1998) 6004–6013.

[9] S. Popov, M. Rexach, L. Ratner, G. Blobel, M. Bukrinsky, Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex, J. Biol. Chem. 273 (1998) 13347–13352.

[10] J. He, S. Choe, R. Walker, P. Di Marzio, D.O. Morgan, N.R. Landau, Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity, J. Virol. 69 (1995) 6705–6711.

[11] J.B. Jowett, V. Planelles, B. Poon, N.P. Shah, M.L. Chen, I.S. Chen, The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle, J. Virol. 69 (1995) 6304–6313.

[12] F. Re, D. Braaten, E.K. Franke, J. Luban, Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B, J. Virol. 69 (1995) 6859–6864.

[13] M.E. Rogel, L.I. Wu, M. Emerman, The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection, J. Virol. 69 (1995) 882–888.

[14] B. Poon, K. Grovit-Ferbas, S.A. Stewart, I.S. Chen, Cell cycle arrest by Vpr in HIV-1 virions and insensitivity to antiretroviral agents, Science 281 (1998) 266–269.

[15] M. Hrimech, X.J. Yao, F. Bachand, N. Rougeau, E.A. Cohen, Human immunodeficiency virus type 1 (HIV-1) Vpr functions as an immediate-early protein during HIV-1 infection, J. Virol. 73 (1999) 4101–4109.

[16] W.C. Goh, M.E. Rogel, C.M. Kinsey, S.F. Michael, P.N. Fultz, M.A. Nowak, B.H. Hahn, M. Emerman, HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo, Nat. Med. 4 (1998) 65–71.

[17] V. Ayyavoo, A. Mahboubi, S. Mahalingam, R. Ramalingam, S. Kudchodkar, W.V. Williams, B.H. Hahn, M. Emerman, HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor kappa B, Nat. Med. 3 (1997) 1117–1123.

[18] M. Nishizawa, M. Kamata, T. Mojin, Y. Nakai, Y. Aida, Induction of apoptosis by the Vpr protein of human immunodeficiency virus type 1 occurs independently of G(2) arrest of the cell cycle, Virology 276 (2000) 16–26.

[19] E. Jacotot, L. Ravagnan, M. Loeffler, K.F. Ferri, H.L. Vieira, N. Zamzami, P. Costantini, S. Druillennec, J. Hoebeke, J.P. Briand, T. Irinopoulou, E. Daugas, S.A. Susin, D. Cointe, Z.H. Xie, J.C. Reed, B.P. Roques, G. Kroemer, The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore, J. Exp. Med. 191 (2000) 33–46.

[20] S.A. Stewart, B. Poon, J.Y. Song, I.S. Chen, Human immunodeficiency virus type 1 vpr induces apoptosis through caspase activation, J. Virol. 74 (2000) 3105–3111.

[21] K. Muthumani, D.S. Hwang, B.M. Desai, D. Zhang, N. Dayes, D.R. Green, D.B. Weiner, HIV-1 Vpr induces apoptosis through caspase 9 in T cells and peripheral blood mononuclear cells, J. Biol. Chem. 277 (2002) 37820–37831.

[22] T. Roumier, H.L. Vieira, M. Castedo, K.F. Ferri, P. Boya, K. Andreau, S. Druillennec, N. Joza, J.M. Penninger, B. Roques, G. Kroemer, The C-terminal moiety of HIV-1 Vpr induces cell death via a caspase-independent mitochondrial pathway, Cell Death Differ. 9 (2002) 1212–1219.

[23] C.M. de Noronha, M.P. Sherman, H.W. Lin, M.V. Cavrois, R.D. Moir, R.D. Goldman, W.C. Greene, Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr, Science 294 (2001) 1105–1108.

[24] J.F. Caceres, A.R. Kornblihtt, Alternative splicing: multiple control mechanisms and involvement in human disease, Trends Genet. 18 (2002) 186–193.

[25] A.I. Lamond, D.L. Spector, Nuclear speckles: a model for nuclear organelles, Nat. Rev. Mol. Cell Biol. 4 (2003) 605–612.

[26] Y. Lu, X.Y. Qian, R.M. Krug, The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing, Genes Dev. 8 (1994) 1817–1828.

[27] P. Fortes, A. Beloso, J. Ortin, Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport, EMBO J. 13 (1994) 704–712.

[28] M.A. Hardwicke, R.M. Sandri-Goldin, The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection, J. Virol. 68 (1994) 4797–4810.

[29] W.R. Hardy, R.M. Sandri-Goldin, Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect, J. Virol. 68 (1994) 7790–7799.

[30] H.E. Bryant, S.E. Wadd, A.I. Lamond, S.J. Silverstein, J.B. Clements, Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step, J. Virol. 75 (2001) 4376–4385.

[31] A. Lindberg, J.P. Kreivi, Splicing inhibition at the level of spliceosome assembly in the presence of herpes simplex virus protein ICP27, Virology 294 (2002) 189–198.

[32] M.H. Malim, J. Hauber, S.Y. Le, J.V. Maizel, B.R. Cullen, The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA, Nature 338 (1989) 254–257.

[33] U. Fischer, J. Huber, W.C. Boelens, I.W. Mattaj, R. Luhrmann, The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs, Cell 82 (1995) 475–483.

[34] M. Fornerod, M. Ohno, M. Yoshida, I.W. Mattaj, CRM1 is an export receptor for leucine-rich nuclear export signals, Cell 90 (1997) 1051–1060.

[35] M. Neville, F. Stutz, L. Lee, L.I. Davis, M. Rosbash, The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export, Curr. Biol. 7 (1997) 767–775.

[36] S. Nakielny, G. Dreyfuss, Transport of proteins and RNAs in and out of the nucleus, Cell 99 (1999) 677–690.
[37] Y. Nishino, T. Myojin, M. Kamata, Y. Aida, Human immunodeficiency virus type 1 Vpr gene product prevents cell proliferation on mouse NIH3T3 cells without the G2 arrest of the cell cycle, Biochem. Biophys. Res. Commun. 232 (1997) 550–554.
[38] M. Nishizawa, T. Myojin, Y. Nishino, Y. Nakai, M. Kamata, Y. Aida, A carboxy-terminally truncated form of the Vpr protein of human immunodeficiency virus type 1 retards cell proliferation independently of G(2) arrest of the cell cycle, Virology 263 (1999) 313–322.
[39] M. Kawamura, T. Ishizaki, A. Ishimoto, T. Shioda, T. Kitamura, A. Adachi, Growth ability of human immunodeficiency virus type 1 auxiliary gene mutants in primary blood macrophage cultures, J. Gen. Virol. 75 (Pt. 9) (1994) 2427–2431.
[40] S. Iijima, Y. Nitahara-Kasahara, K. Kimata, W. Zhong Zhuang, M. Kamata, M. Isogai, M. Miwa, Y. Tsunetsugu-Yokota, Y. Aida, Nuclear localization of Vpr is crucial for the efficient replication of HIV-1 in primary CD4+ T cells, Virology 327 (2004) 249–261.
[41] J.K. Yee, A. Miyanohara, P. LaPorte, K. Bouic, J.C. Burns, T. Friedmann, A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes, Proc. Natl. Acad. Sci. USA 91 (1994) 9564–9568.
[42] A. Mayeda, A.R. Krainer, Mammalian in vitro splicing assays, Methods Mol. Biol. 118 (1999) 315–321.
[43] K.A. Lee, M.R. Green, Small-scale preparation of extracts from radiolabeled cells efficient in pre-mRNA splicing, Methods Enzymol. 181 (1990) 20–30.
[44] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[45] M. Nishizawa, M. Kamata, R. Katsumata, Y. Aida, A carboxy-terminally truncated form of the human immunodeficiency virus type 1 Vpr protein induces apoptosis via G(1) cell cycle arrest, J. Virol. 74 (2000) 6058–6067.
[46] D.N. Levy, L.S. Fernandes, W.V. Williams, D.B. Weiner, Induction of cell differentiation by human immunodeficiency virus 1 vpr, Cell 72 (1993) 541–550.
[47] Y. Qiu, M. Nemeroff, R.M. Krug, The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6-U2 and U6-U4 snRNA interactions during splicing, RNA 1 (1995) 304–316.
[48] K.S. Sciabica, Q.J. Dai, R.M. Sandri-Goldin, ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation, EMBO J. 22 (2003) 1608–1619.
[49] H. de Rocquigny, A. Caneparo, T. Delaunay, J. Bischerour, J.F. Mouscadet, B.P. Roques, Interactions of the C-terminus of viral protein R with nucleic acids are modulated by its N-terminus, Eur. J. Biochem. 267 (2000) 3654–3660.
[50] K. Muthumani, A.Y. Choo, D.S. Hwang, M.A. Chattergoon, N.N. Dayes, D. Zhang, M.D. Lee, U. Duvvuri, D.B. Weiner, Mechanism of HIV-1 viral protein R-induced apoptosis, Biochem. Biophys. Res. Commun. 304 (2003) 583–592.
[51] D. Bentley, The mRNA assembly line: transcription and processing machines in the same factory, Curr. Opin. Cell Biol. 14 (2002) 336–342.
[52] Y.W. Fong, Q. Zhou, Stimulatory effect of splicing factors on transcriptional elongation, Nature 414 (2001) 929–933.
[53] T. Kino, G.N. Pavlakis, Partner molecules of accessory protein Vpr of the human immunodeficiency virus type 1, DNA Cell Biol. 23 (2004) 193–205.
[54] F. Del Gatto-Konczak, C.F. Bourgeois, C. Le Guiner, L. Kister, M.C. Gesnel, J. Stevenin, R. Breathnach, The RNA-binding protein TIA-1 is a novel mammalian splicing regulator acting through intron sequences adjacent to a 5' splice site, Mol. Cell. Biol. 20 (2000) 6287–6299.
[55] P. Forch, O. Puig, N. Kedersha, C. Martinez, S. Granneman, B. Seraphin, P. Anderson, J. Valcarcel, The apoptosis-promoting factor TIA-1 is a regulator of alternative pre-mRNA splicing, Mol. Cell 6 (2000) 1089–1098.
[56] M. Roshal, B. Kim, Y. Zhu, P. Nghiem, V. Planelles, Activation of the ATR-mediated DNA damage response by the HIV-1 viral protein R, J. Biol. Chem. 278 (2003) 25879–25886.
[57] X. Liu, A. Mayeda, M. Tao, Z.M. Zheng, Exonic splicing enhancer-dependent selection of the bovine papillomavirus type 1 nucleotide 3225 3' splice site can be rescued in a cell lacking splicing factor ASF/SF2 through activation of the phosphatidylinositol 3-kinase/Akt pathway, J. Virol. 77 (2003) 2105–2115.
[58] N.A. Patel, C.E. Chalfant, J.E. Watson, J.R. Wyatt, N.M. Dean, D.C. Eichler, D.R. Cooper, Insulin regulates alternative splicing of protein kinase C beta II through a phosphatidylinositol 3-kinase-dependent pathway involving the nuclear serine/arginine-rich splicing factor, SRp40, in skeletal muscle cells, J. Biol. Chem. 276 (2001) 22648–22654.
[59] T.O. Tange, C.K. Damgaard, S. Guth, J. Valcarcel, J. Kjems, The hnRNP A1 protein regulates HIV-1 tat splicing via a novel intron silencer element, EMBO J. 20 (2001) 5748–5758.
[60] A.M. Zahler, C.K. Damgaard, J. Kjems, M. Caputi, SC35 and heterogeneous nuclear ribonucleoprotein A/B proteins bind to a juxtaposed exonic splicing enhancer/exonic splicing silencer element to regulate HIV-1 tat exon 2 splicing, J. Biol. Chem. 279 (2004) 10077–10084.
[61] B.A. Amendt, D. Hesslein, L.J. Chang, C.M. Stoltzfus, Presence of negative and positive cis-acting RNA splicing elements within and flanking the first tat coding exon of human immunodeficiency virus type 1, Mol. Cell. Biol. 14 (1994) 3960–3970.
[62] S. Jacquenet, A. Mereau, P.S. Bilodeau, L. Damier, C.M. Stoltzfus, C. Branlant, A second exon splicing silencer within human immunodeficiency virus type 1 tat exon 2 represses splicing of Tat mRNA and binds protein hnRNP H, J. Biol. Chem. 276 (2001) 40464–40475.
[63] B.R. Cullen, Nuclear mRNA export: insights from virology, Trends Biochem. Sci. 28 (2003) 419–424.
[64] M. Caputi, A. Mayeda, A.R. Krainer, A.M. Zahler, hnRNP A/B proteins are required for inhibition of HIV-1 pre-mRNA splicing, EMBO J. 18 (1999) 4060–4067.

3. RNA工学プラットフォーム
4）生体内RNAプロセシング（RNase P/tRNase ZL誘導型ガイドRNAテクノロジー）

黒崎直子・羽生勇一郎・高久　洋

生体内プロセシング酵素はtRNA前駆体を切断することにより，RNAの機能を制御するものである。この酵素にはリボヌクレアーゼP（RNase P）やtRNase Zなどがある。これらは，基質RNAと特異的に結合する特定の二次構造を有するRNAを認識し，基質RNAの5'末端または3'末端を切断する。この機能を利用して，既知の疾患遺伝子のRNAを切断し，その発現を特異的に抑制することで新しい治療法の開発が期待できる。このように，生体内プロセシング酵素は遺伝子の機能を追求するという目的だけに利用されるのではなく，疾患遺伝子の発現抑制という医学的な役割も十分期待される。

はじめに

これまで知られている生体触媒は物質レベルで4つのタイプ（Ⅰ～Ⅳ）に分類される[1]。タイプⅠはタンパク質だけで触媒活性をもっているタンパク質型である。これには，硫酸エステル，リン酸エステル，ペプチド，DNA，RNAなどの分解を触媒する各種加水分解酵素がある。中には補酵素を必要とするものもあるが，ほとんどがタンパク質だけで触媒活性をもっている。タイプⅡはタンパク質-RNA（補酵素）型で，加水分解酵素以外のすべての酵素が含まれる。具体的には，生体物質の酸化還元反応を触媒する酸化還元酵素，二重結合を残す反応を触媒するリアーゼ，異性体間の転換反応を触媒する転移酵素，生体物質の合成反応を合成する合成酵素などがある。また，補酵素は小さいながら触媒反応には積極的に関与している。タイプⅢはRNA型で，これだけで触媒活性をもっている。例えば，自己スプライシングするRNAやウイロイドなどがこのタイプに属する。最後のタイプⅣはRNA-タンパク質複合体であり，tRNAが機能をもつ分子に成熟する過程に関与するリボヌクレアーゼPなどである。これは，RNA側に触媒活性があり，タンパク質側には触媒活性がなく，RNAにタンパク質が加わって効率が上がるものである。

Ⅰ．RNase PとtRNase Z

特定の遺伝子機能を解明するためには，その遺伝子の発現をいかに制御するかが重要なポイントである。これまでの遺伝子発現制御の方法としては，主にアンチセンス法やリボザイムなどが用いられていたが，最近ではRNAの干渉作用を利用したRNAi（RNA interference）法を用いて遺伝子の発現を制御することで，その機能を解明する研究が盛んに行われている。

key words
RNase P，tRNase Z，リボザイム，アンチセンス法，RNAi，EGSs，sgRNA，HIV-1

これらの手法はすべて，合成核酸が直接標的遺伝子に作用することで遺伝子発現制御を行うものであるが，リボヌクレアーゼP（RNase P）やtRNase ZはRNAとタンパク質の複合体によりその発現を制御することができる。

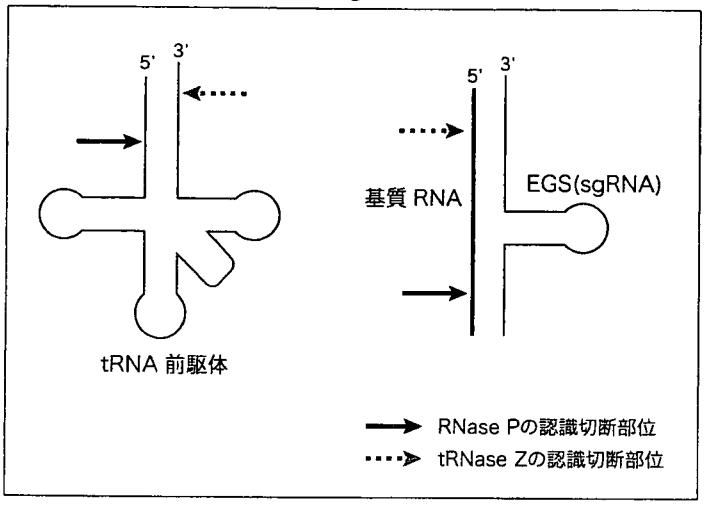
tRNAは，その対応する遺伝子からtRNA前駆体として転写された後，5'と3'の両端が切りそろえられ成熟tRNAとなる。RNase Pは，そのtRNA前駆体の5'側を切断するエンドヌクレアーゼである（図❶）。RNase Pは色々な生物種に広く分布しており，これまでに大腸菌や枯草菌などの細菌，好熱菌・好酸菌・好塩基菌などの古細菌，酵母やヒトなどの真核生物にもその存在が確認されている。

RNase Pが生体内でエンドヌクレアーゼ活性を示すためには，RNA成分とタンパク質成分の両者が必要であると考えられていたが，1983年にAltmanらが，大腸菌や枯草菌のRNase PのRNA成分は試験管内の反応で前駆体tRNAを特定の部位で切断できること，またヌクレアーゼタンパク質には切断活性がないことを発見した[2]。

RNase PはtRNA前駆体のヌクレオチドのみを切断し，他のヌクレオチドは切断しない性質をもっている。また，tRNA前駆体は基質となるヌクレオチド配列が異なるにもかかわらず，これらを認識して特異的に切断することができる。ただし，変異を起こして立体的に正常な構造がとれないtRNA前駆体の場合は，切断速度が非常に遅くなる。

RNase Pの二次構造の共通性は，tRNA前駆体と結合して切断するためとタンパク質成分との結合に必要なために，保存されていると考えられる。細胞内では，タンパク質が一般に触媒として使われている。タンパク質がRNAを認識して結合し，触媒作用を現す。自己スプライシングするリボザイムの場合は他の因子を要求せず，自分自身で自己の切断・連結を行う分子内自己触媒である。しかし，RNase Pの場合は分子間の反応を触媒するので，真の酵素である。

図❶ tRNA前駆体とEGS（sgRNA）の作用機序

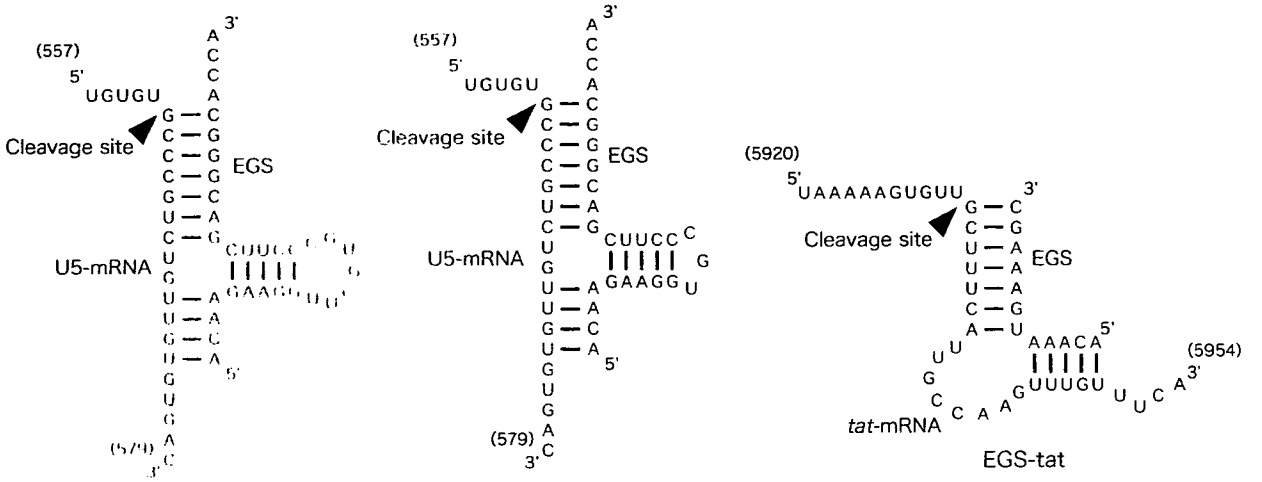


一方，1991年に梨本らはマウスFM3A細胞抽出物より，試験管内においてスペルミジン存在下で配列特異的にRNAを切断する酵素であるtRNaze Z（tRNA 3' processing endoribonuclease）が哺乳動物細胞中にもあることを発見した[3,4]。その後，tRNase ZはtRNA前駆体（67残基のtRNA^{Asp}，66残基のtRNA^{Ala}）の3'末端を特異的に切断することや，ほとんどの哺乳類の細胞に存在し，RNA-タンパク質複合体によって基質RNAのCCA配列下流を切断することがわかってきた[5-7]。tRNase ZはELAC1/ELAC2ファミリーに属し，そのうちELAC1タンパク質は300～400残基のアミノ酸からなるtRNase ZSで，原核細胞・真核細胞などに存在する。一方，ELAC2タンパク質は800～900残基のアミノ酸からなるtRNase ZLで，これは真核細胞のみに存在している[8,9]。

Ⅱ．RNase PとtRNase ZLの新たな遺伝子治療法への応用

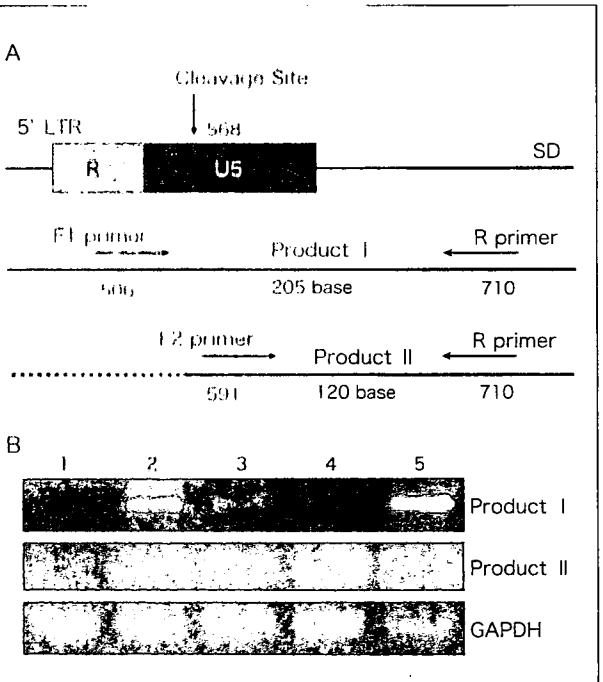
RNA分子（external guide sequences：EGSs）が標的遺伝子（基質RNA）と結合することによって形成されるRNAの二次構造は，tRNAに非常に類似した構造を形成する。そして生体内のヌクレアーゼであるRNase PやtRNase ZLがこれらの構造を認識し，基質RNA部分が切断される。RNase Pは基質RNAの5'側を切断し，tRNase ZLは基質RNAの3'側を切断する。

図❷　EGS（赤字部分）と標的配列（黒字部分）



（グラビア頁参照）

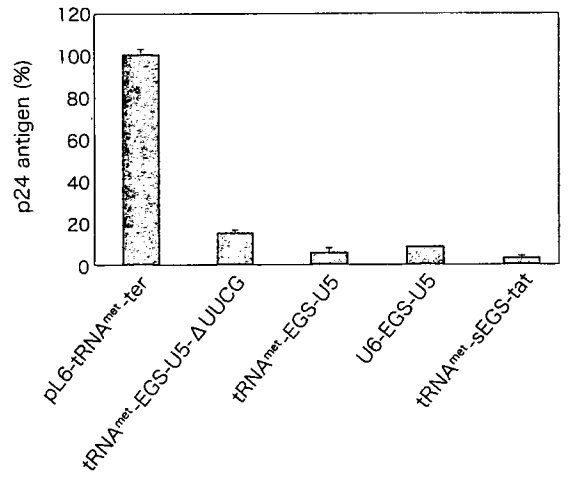
図❸　RNase Pによる標的RNAの切断をRT-PCR法にて検出



A. RT-PCRのプライマー設定位置。Product Iは全長，Product IIは切断部位下流のRNAを検出するプライマー。

B. レーン1：EGS非導入
レーン2：コントロールベクター
レーン3：EGS-U5-ΔUUCG
レーン4：EGS-U5
レーン5：EGS-tat
を導入した際の標的RNAの検出結果

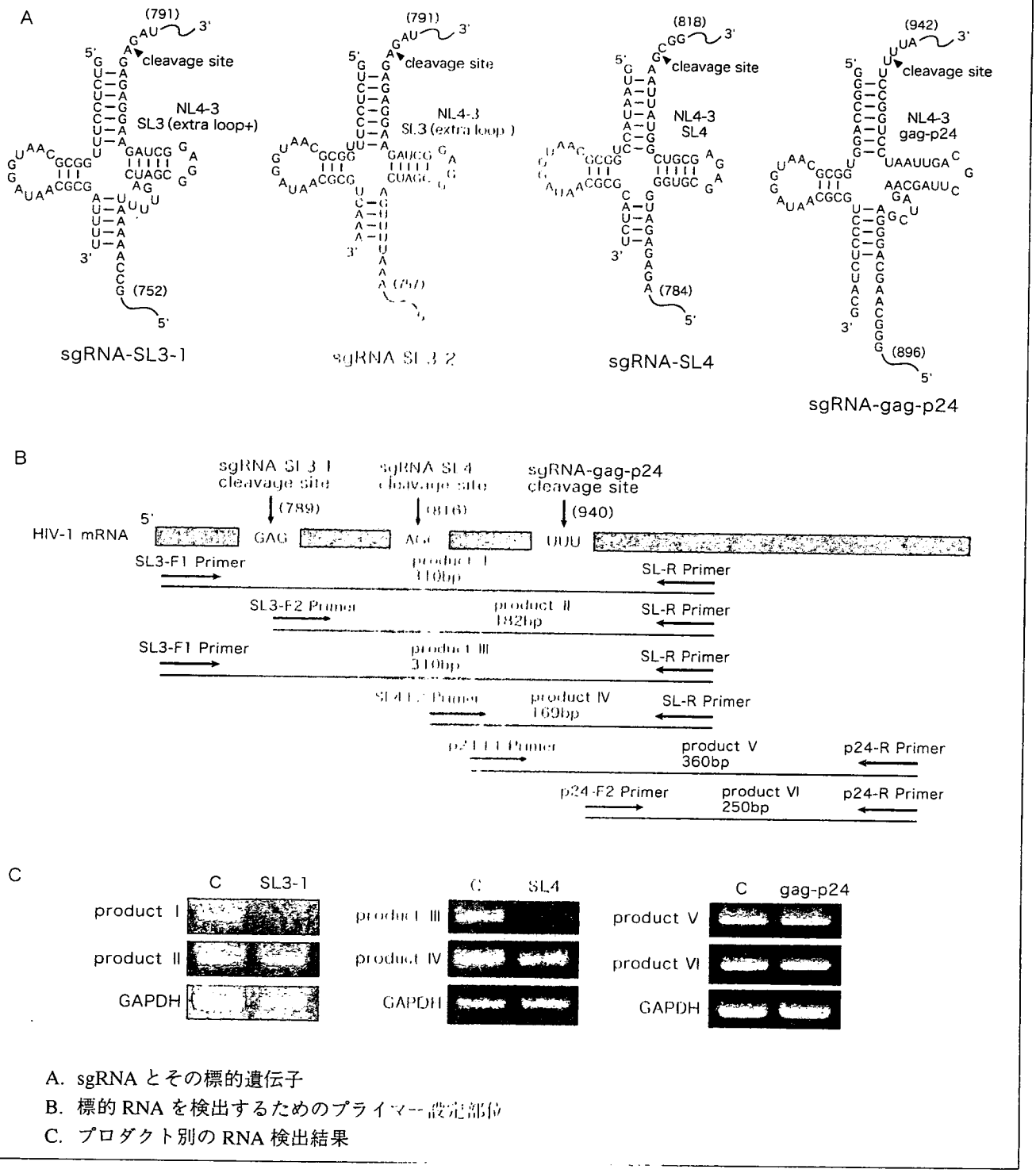
図❹　ウイルスタンパク質発現量



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。

これらの機能を利用して，エイズ感染症の病原ウイルスであるHIV-1（human immunodeficiency virus type-1）[用解1]を基質RNAとし，このウイルスRNAの一部を特異的に認識するEGSsを作製して，RNase PとtRNase ZLを用いてウイルスRNAを切断することで，ウイルスタンパク質の発現制御を試みた。本稿ではその結果を示す。

図❺ HIV-1 に対する sgRNA とウイルス RNA の切断



A. sgRNA とその標的遺伝子
B. 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

Ⅲ. RNase P によるウイルス遺伝子の発現制御[10]

RNase P で切断する標的遺伝子は，HIV-1 の 5' 側 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❷）。EGSs は核内にとどまらないように，tRNA プロモーターまたは U6 プロモーターを利用して，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，ウイルスタンパク質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-1 RNAと結合し，RNase Pによって標的HIV-1 RNAが切断されることをRT-PCR法にて確認した。図❸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のみが検出されたことから，EGSsと標的HIV-1のRNAとの結合によるtRNA様構造をRNase Pが認識し，標的HIV-1のRNAが切断されたことが示唆された。また，この時の培養上清中におけるウイルスタンパク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も確認された（図❹）。

ここで非常に興味深いのは，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❷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タンパク質の発現を抑制することが示唆された。

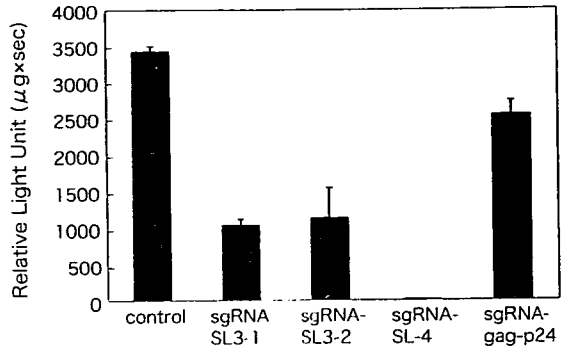
Ⅳ. tRNase ZLによるウイルス遺伝子の発現制御

次に，筆者らがtRNase ZLを利用してHIV-1の発現を制御した例を紹介する[11]。

tRNase ZLはsmall-guide RNA（sgRNA）と標的RNAが作るtRNA前駆体様の構造を認識して，標的RNAの3'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た場合においてウイルス遺伝子を切断するか否かについて検討した。ここで標的としたウイルス遺伝子は，sgRNAが結合した時にtRNA前駆体様の構造をとることが予測される5'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ー遺伝子を組み込んだpNL-lucベクターとともに細胞に導入し，ウイルス由来のタンパク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❺）。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❻）。また，tRNase ZLによるウイルス遺伝子の切断は，ウイルスタンパク質の発現を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。

図❻　ウイルス由来タンパク質の発現量を定量



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）はウイルス由来タンパク質の発現を強く抑制した。

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が実現すれば，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。

用語解説

1. **HIV-1**：AIDS（acquired immunodeficiency syndrome）の病因ウイルス。AIDS は 1981 年にアメリカで最初に報告された後天性免疫不全症候群で，1983 年にその原因ウイルスとして human immunodeficiency virus（HIV）が同定された。HIV-1 はレンチウイルス科に属し，1 型と 2 型がある。2 型（HIV-2）の流行地が西アフリカやインドの一部，ヨーロッパの一部であるのに対して，1 型（HIV-1）は全世界で流行している。

参考文献

1) 柳川弘志：RNA 学のすすめ，講談社，1990.
2) Robertson HD, Altman S, et al : J Biol Chem 247, 5243-5251, 1972.
3) Nashimoto M, Sakai M, et al : Biochem Biophys Res Commun 178, 1247-1252, 1991.
4) Nashimoto M : Nucleic Acids Res 20, 3737-3742, 1992.
5) Nashimoto M : Nucleic Acids Res 23, 3642-3647, 1995.
6) Nashimoto M : Nucleic Acids Res 21, 4696-4702, 1993.
7) Nashimoto M, Kominami R : Biochem Biophys Res Commun 176, 1163-1169, 1991.
8) Takaku H, Minagawa A, et al : Nucleic Acids Res 32, 4429-4438, 2004.
9) Schiffer S, Rosch S, et al : EMBO J 21, 2769-2777, 2002.
10) Banor JS, Miyano-Kurosaki N, et al : Bioorg Med Chem Lett 14, 4941-4944, 2004.
11) Habu Y, Miyano-Kurosaki N, et al : Nucleic Acids Res 33, 235-243, 2005.

参考図書

・ Handbook of Experimental Pharmacology, Erdmann VA, Brosius J, et al ed, Springer, 151-171, 2005.

参考ホームページ

・ 千葉工業大学 工学部生命環境科学科
http://www.it-chiba.ac.jp/faculty/eng/lifescience.html
・ NIDA（National Institute on Drug Abuse）
http://www.drugabuse.gov/NIDAHome.html

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アンチセンス核酸を用いた抗 HIV-1 剤の開発およびウイルスに対する免疫治療法の開発に関する研究

Transworld Research Network
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

RNAi Therapeutics, 2006: 69-78 ISBN: 81-7895-245-9
Editors: Hiroshi Takaku and Naoki Yamamoto

6

Short hairpin RNA synthesized by phage polymerase do not induce interferon in hepatitis C virus subgenomic replicons

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Abstract

RNA interference (RNAi) is a cellular process that induces gene silencing by which small duplexes of RNA specifically target a homologous sequence for cleavage by cellular ribonucleases. Here, to test the RNAi method for blocking hepatitis C virus (HCV) RNA replication, we created four short hairpin RNAs (shRNAs) targeting the HCV internal ribosome entry site/Core gene transcript using T7 RNA polymerase.

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shRNA suppressed the replication of HCV RNA in the HCV replicon. On the other hand, short interfering RNAs synthesized using the T7 RNA polymerase system trigger a potent induction of interferon-α and -β in a variety of cells. We examined whether the shRNAs synthesized using the T7 RNA polymerase system activated double-stranded RNA-dependent protein kinase, 2'-5' oligoadenylate synthetase, or interferon-regulatory factor-3. Our results demonstrated that the T7-transcribed shRNA did not activate these proteins in Huh-7 cells and the HCV replicon. These shRNAs are a promising new strategy for anti-HCV gene therapeutics.

Introduction

RNA interference (RNAi) occurs in a variety of organisms, including Caenorhabditis elegans [1], Trypanosoma brucei [2], plants [3], Drosophila [4], planaria [5], zebrafish [6], and mouse embryos [7]. In most of these organisms, the injection of a double-stranded RNA (dsRNA) longer than 500 bp specifically suppresses the expression of the gene with the corresponding DNA sequence, but has no effect on genes with unrelated sequences.

RNAi is initiated by the RNase III-like nuclease Dicer, which promotes progressive cleavage of long dsRNAs into 21 to 27 nucleotide (nt) short interfering RNAs (siRNAs) with two nt 3'-overhangs. 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 [8-10].

Hepatitis C virus (HCV) is one of the main causes of liver-related morbidity and mortality [11]. The virus establishes a persistent infection in the liver, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinomas [11]. HCV replication occurs in the cytoplasm and is associated with membranes that appear to be derived from the endoplasmic reticulum. Genomic HCV RNA is translated to produce a 3000-amino acid polypeptide that is processed into at least 10 proteins. The nonstructural proteins 3, 4A, 4B, 5A, and 5B form a replicase complex that promotes transcription of a genomic (-) strand intermediate. This serves as a template for the production of (+) strands that are either translated or packaged into virions as genomic RNAs [12, 13]. A satisfactory treatment for HCV infection has yet to be developed, however, because studies of HCV have been hampered by the lack of a stable cell-culture system and a small animal model. One recently reported HCV replicon is a selectable sub-genomic HCV RNA, which replicates efficiently and continuously in human hepatoma Huh-7 cells [14, 15]. HCV RNA replication is also sensitive to RNAi [16-18].

On the other hand, it was previously reported that dsRNA triggers the production of type I interferon (IFN), and activates dsRNA-dependent protein kinase (PKR) [19] and 2'-5'oligoadenylate synthetase (2'-5'OAS). Furthermore, two recent studies demonstrated that the mechanism of the IFN response might include recognition of the siRNAs by Toll-like receptor-3 (TLR-3) [20]. One simple method for limiting the risk of inducing an IFN response is to use the lowest effective dose of short hairpin RNA (shRNA) vector, as advocated by Bridge et al. [21]. Recently, Kim et al. reported that siRNAs synthesized using the T7 RNA polymerase system can trigger the potent induction of IFN-α and -β in a variety of cells [22]. These results demonstrated an association between the sequence specific-inhibition via the RNAi mechanism without stimulating the TLR-3 signal pathway, PKR [23], or 2'-5'OAS by HCV proteins, because the TLR-3-adaptor protein TRIF is cleaved by HCV nonstructural 3/4A protease [24]. On the other hand, the nonstructural 5A protein might bind with PKR and block dimerization, which inhibits the activation of eukaryotic initiation factor 2α [25, 26].

In the present study, we synthesized four shRNAs targeting the HCV internal ribosome entry site (IRES)/Core gene transcript using T7 RNA polymerase. The greatest inhibitory effects occurred with both HCV 330-349-shRNA and HCV 340-359-shRNA, as the target of the HCV RNA. We also examined whether the shRNAs synthesized using the T7 RNA polymerase system activated PKR, 2'-5'OAS, or IFN-regulatory factor-3 (IRF-3). shRNA synthesized using T7 RNA polymerase did not, however, activate these proteins in Huh-7 cells and HCV replicons.

Inhibition of HCV RNA replication of synthesized shRNAs using T7 RNA polymerase in the HCV replicon

The present study examined whether HCV RNA replication was inhibited by an RNAi mechanism. We synthesized four shRNAs targeting the HCV IRES/Core gene transcript using T7 RNA polymerase (Fig. 1a) and verified the sequences using 18% polyacrylamide gel electrophoresis (data not shown). The sequences are shown in Fig. 1b. To assess the inhibitory effects of the shRNAs on the intracellular replication of HCV, we used HCV replicons. Transfection of the shRNA into the HCV replicons (Fig. 1a), which stably express the HCV subgenome, indicated that the 330-349-shRNA and 340-359-shRNA both inhibited HCV RNA replication in a dose-dependent manner (Fig. 2). On the other hand, the control, EGFP-shRNA, did not induce efficient inhibition (Fig. 2). These findings suggest that targeting the region that includes the AUG of the HCV IRES/Core is effective.