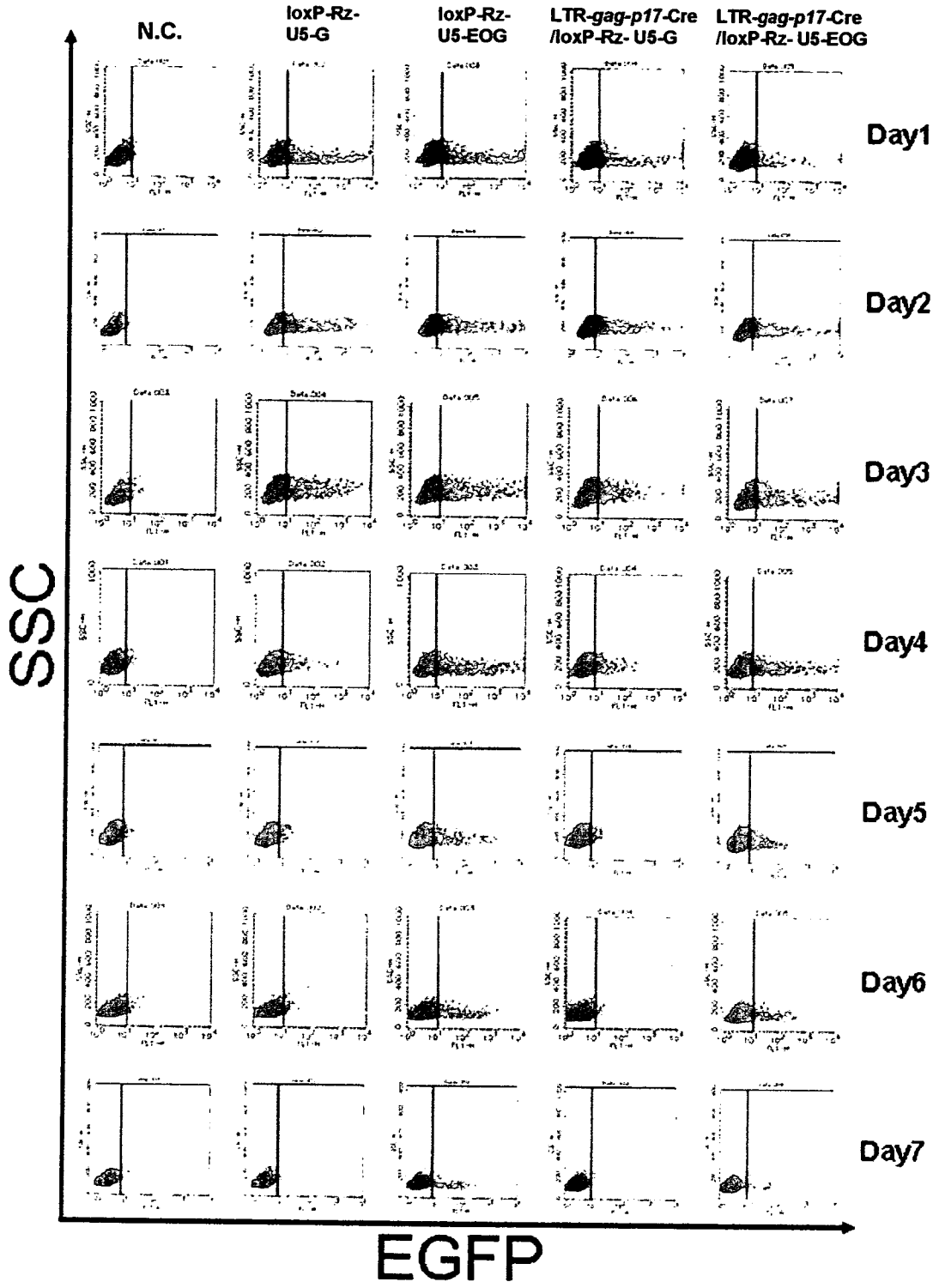

Figure 3. Enhancement of transgene expression by *EBNA-1* and *oriP* in HeLa $CD4^+$ cells. EGFP expression was evaluated on days 1 to 7 post-transfection by flow cytometry.

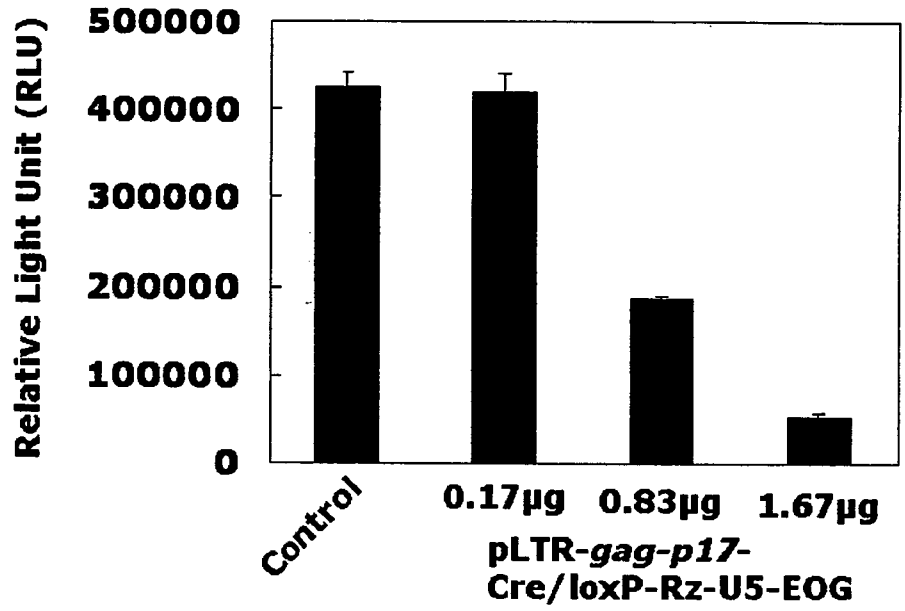

Figure 4. Dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG in HIV-1-transfected cells. Different amounts of pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG (0.17, 0.83 or 1.67 μg) were co-transfected with HIV-1 (pNL4-3-luc) into HeLa $CD4^+$ cells. At 72 h post-transfection, luciferase activity of the cell lysate was measured as an indirect marker for viral replication.

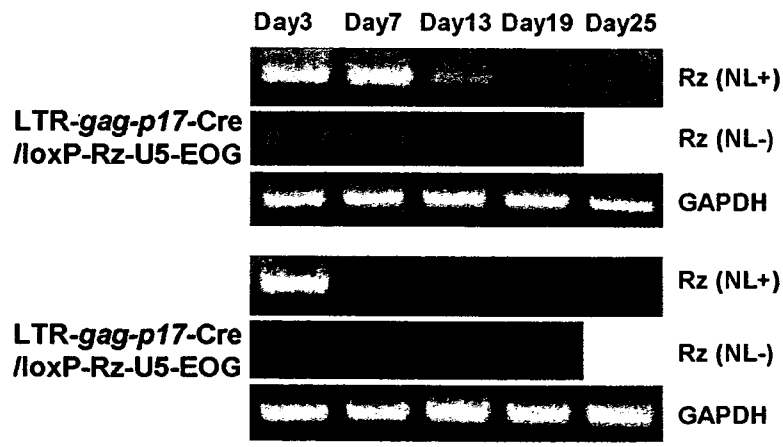

Figure 5. RT-PCR analysis shows persistent ribozyme expression of HeLa $CD4^+$ cells transfected with *EBNA-1/oriP*-containing vector. Total RNA was isolated from confluent pNL4-3 infected cell cultures transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5-G or pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG. RT-PCR using ribozyme-specific primers was performed concurrently with amplification of GAPDH mRNA.

the Cre/loxP vector containing EBNA-1 has the potential to confer long-term transgene expression in HIV-1 infected cells. This property therefore puts our vector system in the class of prophylactics, since the vector will function to prevent infection of the target cells.

Long-term inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5- EOG in pNL4-3 infected HeLa CD4$^+$ cells

To evaluate further the persistency of the EBNA-1/oriP-dependent ribozyme effect on HIV-1 replication we co-transfected 0.3 µg pNL4-3 with 5.0 µg pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG, pLTR- *gag-p17*-Cre/loxP-Rz-U5-G or ploxP-Rz-U5-EOG into HeLa CD4$^+$ cells. As shown in Figure 6A, pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG inhibited HIV-1 replication for 15 days. By contrast, ploxP-Rz-U5-EOG (lacking ribozyme expression) showed little inhibitory activity towards HIV-1 replication compared with the negative control (pNL4-3 only).

EGFP reporter gene expression was monitored as an indicator of transgene expression (Figure 6B). Its persistence throughout the experiment (up to 13 days) confirms our earlier findings, and suggests that the HIV-1-dependent ribozyme-expression vector containing EBNA-1/oriP sequences mediates long-term ribozyme expression, inhibits HIV-1 replication and would be a useful tool for HIV-1 gene-therapy applications.

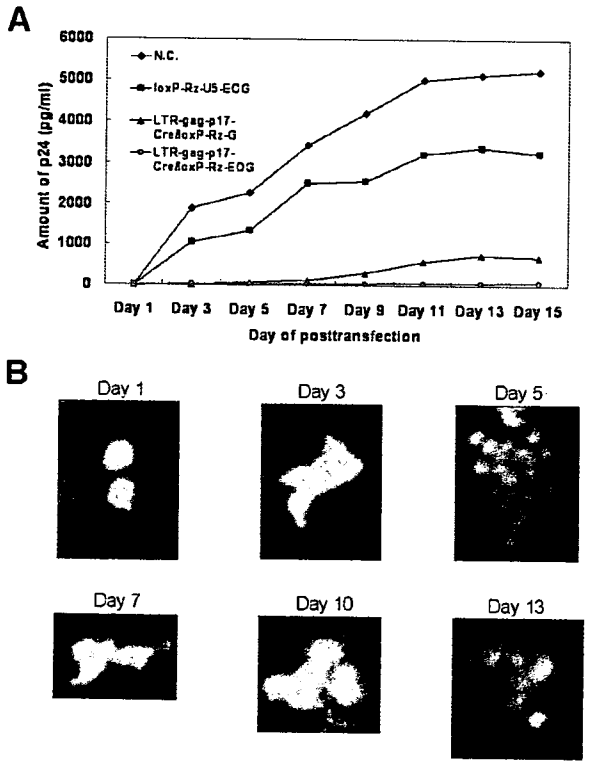

Figure 6. Inhibitory effects on HIV-1 p24 antigen production by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG. **(A)** HeLa CD4$^+$ cells were co-transfected with HIV-1 pNL4-3 and either control vector (diamonds), ploxP-Rz-U5-EOG (squares), pLTR-*gag-p17*-Cre/loxP-Rz-U5-G (triangles), or pLTR-*gag-p17*-Cre/loxP-Rz-U5- EOG (circles). HIV-1-virus production was measured by p24 ELISA at the indicated times post-transfection. **(B)** Transgene replication in dividing cells. Cells were passaged every 2 days, and monitored for transgene replication by way of EGFP expression in singly dividing cells.

CONCLUSIONS

- Ribozyme expression is HIV-1 Tat dependent, as we were only able to detect it in the presence of pNL4-3.
- Transgene expression is long-term and is extended by the presence of oriP and EBNA-1. Hence, inhibition is also shown to be long-term.
- The Cre/loxP system induces ribozyme-mediated inhibition of HIV-1 replication in a dose-dependent manner.
- These results demonstrate the anti-HIV-1 effect of the expressed ribozyme from the Cre/loxP system, clearly illustrating the potential of the Cre/loxP-oriP/EBNA-1 system as a gene-therapy tool for controlling HIV-1 infection.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for High Technology Research (HTR) from the Ministry of Education, Science, Sports, and Culture, Japan, by research grants from the Human Science Foundation (HIV-K-14719), by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare, Japan (H17-AIDS-002), and by the Sasakawa Scientific Research Grant from The Japan Science Society. Y. H. was a Research Fellow of the HTR until June 2005 and has been a Research Fellow of the Japanese Foundation for AIDS Prevention since July 2005.

STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

LIST OF ABBREVIATIONS

EBV; Epstein-Barr virus
EBNA-1; EBV nuclear antigen 1
EOG; EBNA-1/OriP/EGFP
MFI; Mean fluorescence intensity
OriP; EBV latent origin of replication

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Nucleosides, Nucleotides, and Nucleic Acids, 25:427–437, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/01457630600684120

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SUPPRESSION OF HIV-1 REPLICATION BY A COMBINATION OF ENDONUCLEOLYTIC RIBOZYMES (RNase P AND tRNase ZL)

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□ *We examined the combinatorial action of RNase P and tRNase ZL-mediated specific inhibition of HIV-1 in cultured cells. We designed two short extra guide sequences (sEGS) that specifically recognize the tat and vif regions of HIV-1 mRNA and mediate the subsequent cleavage of hybridized mRNA by the RNase P and tRNase ZL components. We constructed an RNase P and tRNase ZL-associated vif and tat sEGS expression vector, which used the RNA-polymerase III dependent U6 promoter, as an expression cassette for EGS. Together, the RNase P and tRNase ZL-associated sEGS molecules allow more efficient suppression of HIV-1 mRNA production when separately applied. The possibilities offered by the vector to encode sEGS will provide a powerful tool for gene therapy.*

Keywords RNase P; tRNase ZL; External guide sequence; HIV-1; Gene therapy

INTRODUCTION

Small inhibitory RNAs (siRNAs) are small RNAs of 21-nucleotide length that trigger the destruction of target mRNA with which they share complementarity.[1] RNAi has emerged as a powerful tool to probe the function of genes of known sequence *in vitro* and *in vivo*. Advances in vector design

Received 1 December 2005; accepted 23 January 2006.

This work was supported in part by a Grant-in-Aid for High Technology Research, from the Ministry of Education, Science, and Culture, Japan, and also by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare, Japan (H17-AIDS-002). Y. H. was a Research Fellow of the HTR until June 2005, and has been a Research Fellow of the Japanese Foundation for AIDS Prevention since July 2005. Both authors Ikeda and Habu contributed equally to this work.

This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.

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permit the effective expression of siRNA in human cells by transfer of short hairpin RNA (shRNA) expression cassettes.[2] Recent investigations have described the ability of RNAi to decrease the replication of human immunodeficiency virus type 1 (HIV-1) in lymphocytic cells using siRNA targeting viral (e.g., tat, gag, rev, nef)[3–7] and host (e.g., CCR5, CXCR4)[8,9] proteins. RNAi can be used as a form of genetic therapy for HIV-1 and associated infections.

When profound inhibition of virus replication is obtained by means of RNAi technology, one has to consider the possibility of viral escape. This potential problem is particularly true for viruses that exhibit significant genetic variation due to an error-prone replication machinery. Thus, this problem may be more severe for RNA viruses and retroviruses than DNA viruses. Indeed, RNAi-resistant poliovirus and HIV-1 variants have already been reported.[10–12]

This problem can be overcome with the catalytic RNA subunit RNase P which can in principle be targeted to cleave any target RNA using the external guide sequences (EGS).[13] This strategy is unique in that cleavage of a specific target mRNA occurs after hybridization of the EGS to form a structure resembling a tRNA substrate.[14,15] RNA-based EGSs have been expressed endogenously as transgenes in both bacteria and mammalian cells,[14,16] and have been effective in inhibiting gene expression by HIV-1.[17,18] Mammalian cells contain the essential enzyme, tRNA 3′-processing endoribonuclease (tRNase Z or 3′-tRNase; EC 3.1.26.11), which removes 3′trailers from pre-tRNAs.[19] The long-form enzyme (tRNase ZL) is unique in that it can cleave any RNA at any site when directed by a small-guide RNA (sgRNA) in vitro.[20–22] Recently, we demonstrated the efficacy of this method in specifically targeting RNA in HIV-1 infected T-cells by introducing sgRNAs encoded by expression retroviral vector. Mo-MLV-based sgRNA-SL4 targeting the HIV-1 gag gene could suppress sgRNA-dependent HIV-1 expression in human T cells.[23]

In this article, we demonstrate the combinatorial action of RNase P and tRNase ZL-mediated specific inhibition of HIV-1 in cultured cells. We designed two truncated short extra guide sequences (sEGS) specifically recognize the tat and vif regions of HIV-1 mRNA and mediate subsequent cleavage of hybridized mRNA by the RNase P and tRNase ZL components. Combination of RNase P and tRNase ZL-associated sEGS molecules allows more efficient suppression of HIV-1 mRNA than separate application.

RESULTS AND DISCUSSION

Design and Construction of the U6–EGS Driven Expression System

In a previous paper, we demonstrated the inhibition of HIV-1 products using sEGS and RNase P to cleave an HIV-RNA target (substrate) in cultured

cells.[18] The short EGS, 12 nucleotides long, was designed to hybridize as an sEGS to a region of the viral RNA with the expected cleavage site located 5′ to the double stranded region (Figure 1B). The greatest inhibitory effect on HIV-1 replication was detected with the sEGS (sEGS-tat) vector as the target of the HIV-1 tat gene (Figures 1A and B). Furthermore, we demonstrated the inhibition of HIV-1 gene products in cultured cells by inducing HIV-1 mRNA cleavage using a modified 5′-half-tRNA^{Arg} (sgRNA) and mammalian tRNase ZL.[23] The greatest inhibitory effect on HIV-1 expression was achieved using sgRNA targeting the HIV-1 gag gene.

In this article, we selected the vif (5521-5533) and tat (5921-5940) as the target sites and tested the RNase P and tRNase ZL-associated sEGS (RNase P-tat-sEGS and tRNase-ZL-vif-sEGS) in one molecule for anti-HIV activity (Figures 1B–D). The complete T loop of RNase P-*tat*-sEGS was replaced by single-stranded sequence UUCA, whereas the T-stem loop of tRNase ZL-vi *f* sEGS included the T-stem loop (5′-CCAGGUUCGACUCCUGG-3′) of wild-type tRNA^{Arg}.

Alternatively, small RNA molecules might also be expressed in the cell following the cloning of siRNA templates into RNA polymerase III (pol III) transcription units, which are based on the sequences of the natural transcription units of the small nuclear RNA U6.[24,25] We cloned the vif-sEGS, tat-sEGS, and vif-tat-sEGS lined with linker (5′-CGCUCGAGU-3′) between either the vif-sEGS-5′ end and tat-sEGS-3′ end genes into Kpn I and BamH I sites downstream of the human U6 snRNA promoter[24] of mammalian expression vector (pSV2neo-U6) (Figure 2). These vectors have been previously described with a high cleavage affinity.[18,23] Furthermore, the control vector designs were constructed to express pSV2neo-U6-mutant-EGS expression vectors (U6-mvif-sEGS, U6-mtat-sEGS, and U6-mvif-mtat-sEGS) (Figure 2).

Expression of the sEGS in Target Cells

To characterize the effect of transge expression, we monitored green-fluorescent protein (GFP) expression in the COS cells transfected with three different plasmids (U6-vif-sEGS, U6-tat-sEGS, and U6-vif-tat-sEGS). GFP expression in these transfected COS cells were observed for 3 days (Figure 3A). To ensure that the therapeutic gene expression by a plasmid vector, pU6-vif-tat-sEGS in target cells, we carried out Northern blot analysis on total RNAs extracted from transduced COS cells. As shown in Figure 3B, a combination of U6-vif-tat-sEGS was expressed at readily detectable levels.

Suppression of HIV-1 Replication by the EGS

To test the ability of the sEGS-expressed by the mammalian expression vectors (pSV2-neo-U6-sEGS) to inhibit HIV-1 replication in a transient assay,

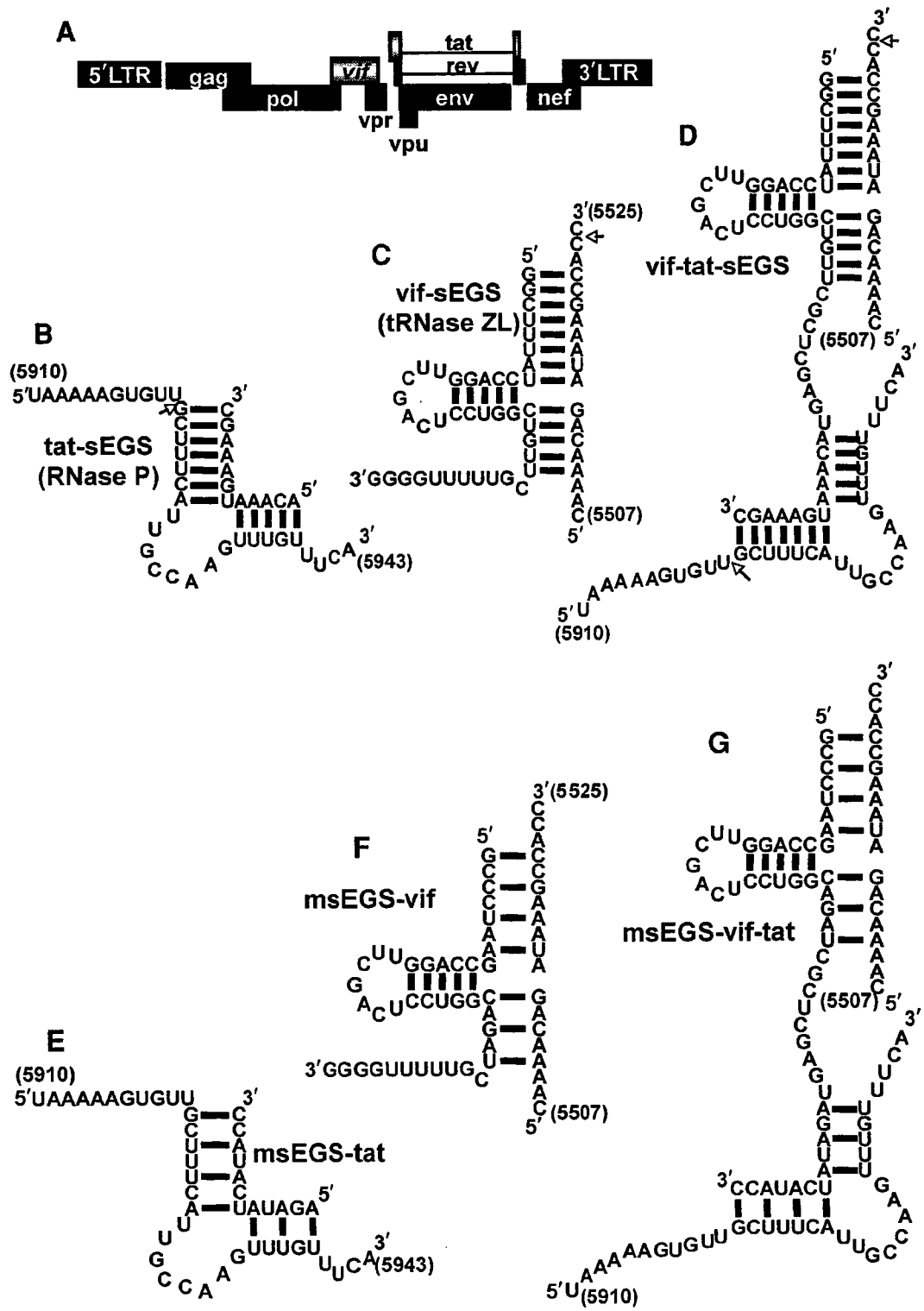

FIGURE 1 Locations of the target sites and secondary structures in the HIV-1 gene of sgRNAs. (A) Locations of the target sites in the HIV-1 gene of sEGS. (B-G) Plausible secondary structures of complexes of the two target sites within the HIV-1 genome (the vif and tat within the HIV-1 gene) with the modified 5′-half-tRNA^{Arg} (tat-sEGS) containing 9 and 5 nt sequences and the T-stem and loop as well as the acceptor stem to the complementary to the target HIV-1 vif site and the tat gene. The arrow indicates the RNase P and tRNase ZL cleavage point.

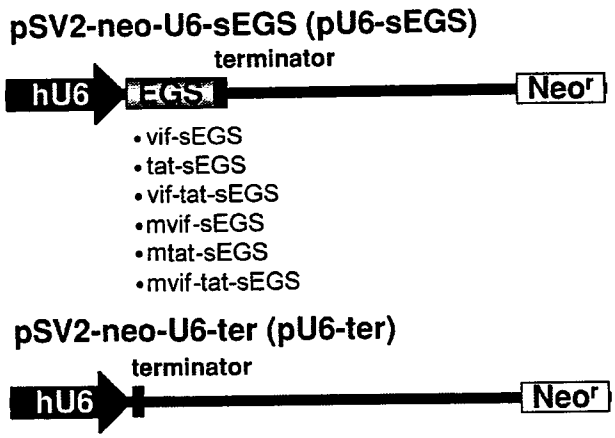

FIGURE 2 Schematic representation of sEGS-expression vectors. The pSVneo-U6-sEGS vectors, pU6-vif-sEGS, pU6-tat-sEGS, pU6-vif-ta-sEGS, pU6-mvif-sEGS, pU6-mtat-sEGS, and pU6-mvif-tat-sEGS. Control vector, pSVneo-U6-ter, lacked sEGS in pSVneo-U6-sEGS.

a viral plasmid (pNL4-3-luc)[26] and the pSV2-neo-U6-sEGSs (U6-vif-sEGS, U6-tat-sEGS, and U6-vif-tat-sEGS) or pSV2-neo-U6-ter (U6-ter) with the U6 promoter and terminator as the control plasmid, were cotransfected into COS cells by the transfection reagent, FuGENE6. The virus production in the culture supernatant was monitored by the HIV-1 p24 antigen (gag gene

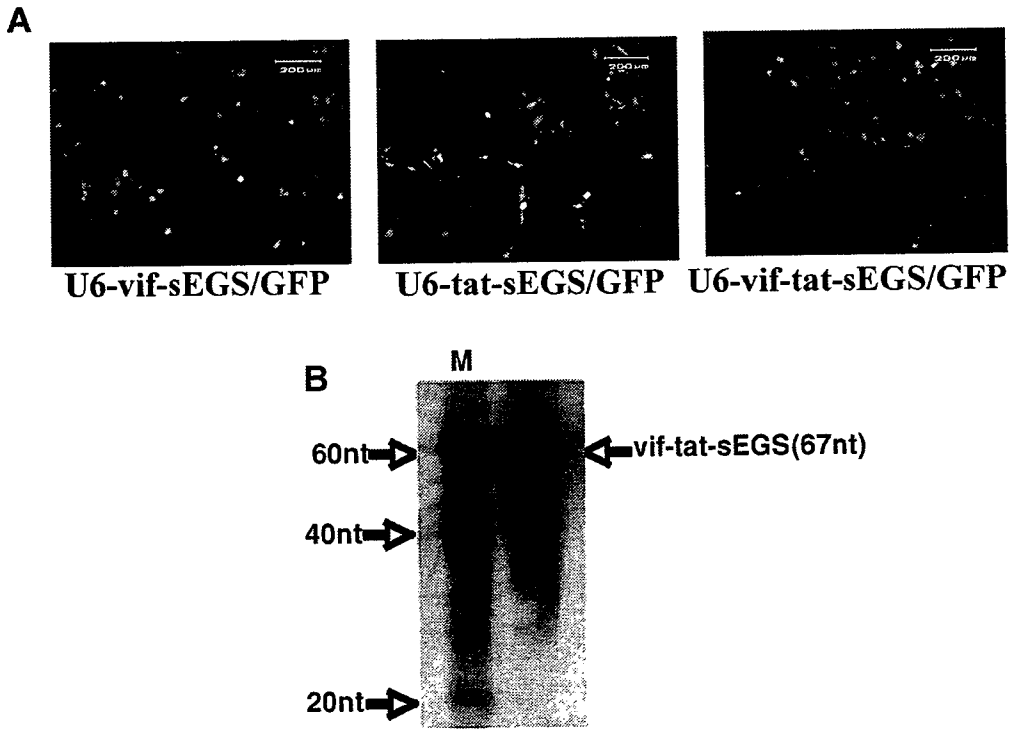

FIGURE 3 Expression of transgenes. (A) To monitor the continuous expression of the transgenes in the transduced cells throughout the culture period, the cells were transferred onto microscope slides and examined for GFP expression during the sampling of the culture cells and supernatants. (B) The presence of RNA was analyzed by northern blotting. Plasmids encoding vif-tat-sEGS were introduced into COS cells. After 72 h, the cells were collected, total RNA was isolated and fractionated on a 15% polyacrylamide gel.

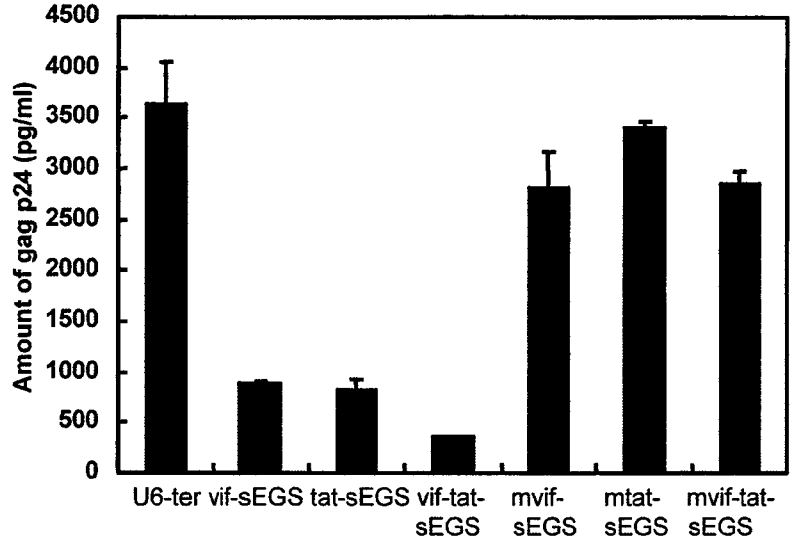

FIGURE 4 Inhibition of production of HIV-1 gag p24 antigen in COS cells transduced with pU6-sEGS. COS cells were transduced with pU6-sEGS and pNL4-3-luc. The pU6-vif-tat-sEGS showed significant suppression of the HIV-1p24 antigen expression in COS cells. Data are representative of three independent experiments.

product) assay.[27] Low levels of p24 product for both U6-vif-sEGS and U6-tat-sEGS used in the challenge assays were detected, and no differences between the EGS efficiencies were observed (Figure 4). In contract, combination of RNase P and tRNase ZL associated sEGSs molecules significantly reduced the level of HIV-1 p24 antigen as compared with that of either U6-vif-sEGS and U6-tat-sEGS (Figure 4). On the other hand, the control sEGSs (U6-mvif-sEGS, U6-mtat-sEGS, and U6-mvif-tat-sEGS) had no inhibitory effect on HIV-1 p24 antigen. These results suggest that the combination of two different antiviral RNAs provides more than separate application.

Effect of a Combination of RNase P and tRNase ZL-Associated sEGS

We also examined the HIV-1 mRNA levels to identify the contribution of HIV-1 mRNA cleavage to the sEGS-mediated anti-HIV-1 effect. The RT-PCR reactions were used to establish the level of uncleaved HIV-1 mRNA (product I and II).[28] The uncleaved HIV-1 mRNA was amplified by the vif-F1and tat-F1 primers, and the vif-R and tat-R primers (Figure 5A). The level of product I and II were expected to decrease after cleavage of the HIV-1 mRNA. The results, which are shown in Figure 5B, indicate that the vif-sEGS and tat-dependent expression system reduced the amount of full-length HIV-1 mRNA (product I and II), whereas COS cells transfected with pNL4-3-luc/mutant-sEGS did not exhibit a significantly altered level of intact HIV-1 mRNA (Figure 5B). The reduction in functional full-length HIV-1 mRNA was consistent with the cleavage effects of vif-sEGS and tat-sEGS at the

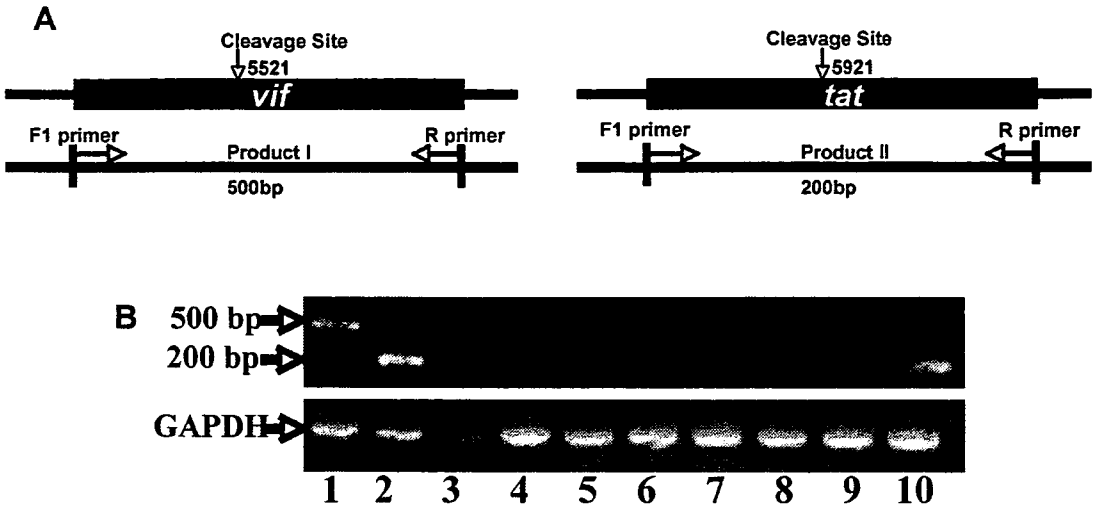

FIGURE 5 RT-PCR analyses of HIV-1 mRNA expression. RT-PCR analyses of uncleaved (product I and II), HIV-1 mRNA were performed using HIV-1 vif and tat-specific primers with concurrent amplification of GAPDH mRNA. (A) Schematic representation of HIV-1-specific primer sites with respect to HIV-1 mRNA: F1 primers, vif and tat; R-primers, vif and tat. (B) RT-PCR amplification products analysed by 2% agarose gel electrophoresis with ethidium bromide staining RT-PCR analysis of HIV-1 mRNA expression in COS cells. RT-PCR analysis of uncleaved HIV-1 mRNA was carried out using HIV-1 specific primers with concomitant amplification of GAPDH mRNA. Lane 1: control vif; lane 2: control tat; lane 3: vif-sEGS; lane 4: tat-sEGS; lane 5: vif-tat-sEGS (vif); lane 6: vif-tat-sEGS (tat); lane 7: mvif-sEGS; lane 8: mtat-sEGS; lane 9: mvif-tat-sEGS (vif); lane 10: mvif-tat-sEGS (tat).

post-transcriptional level. These results indicate that the binding of the sEGS to its target HIV-1 mRNA, and cleavage of pre-tRNA complexes with RNase P and tRNase ZL, might occur in the nucleus. The reduction in functional full-length HIV-1 mRNA was consistent with the RNase P and tRNase ZL cleavage effect at the post-transcriptional level.

This work explores the combination of two inhibitor RNAs (RNase P and tRNase ZL) in one molecule, generating a multifunctional RNA tool, which we have termed an "endonucleolytic ribozymes" (enR). The combination of an RNase P and a tRNase ZL-associated EGSs molecules would be advantageous than separate application. It possible to take advantage of the possibilities offered by the vector to encode sEGS and provides a powerful tool for HIV-1 gene therapy.

EXPERMENTAL PROCEDURES

Cell Cultures

COS and MT-4 cells were grown in complete culture medium consisting of either RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) or D-MEM (Sigma Chemical Co.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cultures were maintained at 37°C under a 5% CO_2 atmosphere.

Construction of U6 Expression Plasmid Vectors

Expression plasmids were constructed using standard techniques. EGS-RNA sequences that were chemically synthesized as two complementary DNA oligonucleotides were mixed in equimolar amounts, heated for 5 min at 95°C, and then gradually cooled down to RT in annealing buffer (10 mM Tris-HCl/100 mM NaCl). The resultant duplex was ethanol precipitated and then ligated into the Kpn I and BamH I cloning sites downstream of the human U6 snRNA promoter of mammalian expression vectors (pSV2neo) (BD Biosciences Clontech, Mountain View, CA) in order to produce the following: the pSV2neo-U6-vif-tat-sEGS vector encoding both HIV-1 vif and tat RNA as dsRNA sense sequences containing KpnI and BamH I cloning sites (5′-GGCTTTATCCAGGTTCGACTCCTGGCTGTT-CGCTCGAGACAAATGAAAGCTTTTTG-3′) and antisense sequences (5′-GATCCCAAAAAGCTTTCATTTCTCTCGAGCGAACAGCCAGGAGTCGAA-CCTGGATAAAGCCGTA-3′); the pSV2neo-U6-vif-sEGS vector encoding HIV-1 vif sense fragment sequences (5′-CGCTTTATCCAGGTTCGACTC-CTGGCTGTTCGTTTTTGGGGTACG-3′) and antisense sequences (5′-GAT-CCGTACCCCAAAAACGAACAGCCAGGAGTCGAACC- TGGATAAAGCCG-TAC-3′); the pSV2neo-U6-tat RNA vector encoding HIV-1 tat sense sequences (5′-ACAAATGAAAGCTTTTTG-3′) and antisense sequences (5′-GATCCAA-AAAGCTTTCATTTGTGTAC-3′); the pSV2neo-U6-mvif-sEGS vector encoding mutated vif sense sequences (5′-CCCCTAAGCCAGGTTCGAC-TCCTGGCAGATCGTTTTTGGGGTACG-3′) and antisense sequences (5′-GATCCGTACCCCAAAAACGATCTGCCAGGAGTCGAACCTGGCTTA-GGCCGTAC-3′); the pSV2neo-U6-mtat-sEGS vector encoding mutated tat sense sequences (5′-AGATATCATACCTTTTTG-3′) and antisense sequences (5′-GATCCAAAAAGGTATGATATCTGTAC-3′); and, finally, the pSV2neo-U6-mvif- tat-sEGS vector encoding mutated vif and tat sense sequences (5′-GCCCTAAGCCAGGTTCGACTCCTGGCAGATCGCTCGAGAGATATC-ATACCTTTTTG-3′) and antisense sequences (5′-GATCCCAAAAAGGTA-TGAATCCTCTCGAGCGATCTGCCAGGAGTCGAACCTGGCTTAGGGCG-TAC-3′); as control expression vectors.

Fluorescent Microscopy

COS cells were grown to ~80% confluence (3×10^5 cells) and transfected with 1 μg each of the sEGS-RNA vectors using 3 μl of FuGENE 6 (Roche Applied Science, Indianapolis, IN). The transfected COS cells were trypsinized, washed twice in PBS, and fixed in PBS containing 1% formaldehyde. Direct fluorescence microscopy of GFP was carried out at the mitotic stage of cell division after each passage on day 3; the data were acquired with a DP12 digital microscope camera (Olympus Company, Tokyo, Japan).

Northern Blot Analysis

Total RNA was extracted with Trizol reagent (GibcoBRL Life Technologies, Carlsbad, CA) from vector-transfected cells, and samples (30 μg) were loaded onto a 20% polyacrylamide/8M urea gel. After electrophoresis, the RNA bands were transferred onto a Hybond-N^{TM} nylon membrane (Amersham Co., Buckinghamshire, UK). The membrane was probed with synthetic oligonucleotides that were complementary to the antisense strand of the env shRNA. Hybridization was carried out at 42°C, and was followed by washing with 2 × SSC at 25°C prior to autoradiography.

Antiviral Assay

To each well of a six-well plate were added COS 3×10^5 cells in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% fetal bovoine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Approximately 24 h after plating, when the cells had reached 80% confluence, sEGS-RNA vectors (1 μg) and pNL4-3-luc (0.1 μg) using 3 μl of FuGENE 6 (Roche Applied Science) were generated according to the manufacturer's optimized protocols. The envelope-defective HIV-1NL4-3-based retroviral vector containing a luciferase expression marker (pNL4-3-luc) was generated by substituting nef gene sequences of the HIV-1NL4-3 genome with the firefly luciferase gene and deleting the envelope gene sequences located between two Bgl II restriction endonuclease sites.[26] After 3 days of incubation, the virus replication was monitored in the cell-free culture supernatants with the HIV-1 p24 CLEIA assay (Lumipulse, Fujirebio Inc., Tokyo, Japan).[27]

Cleavage Activities of sEGS in COS Cells

COS cells were grown to ~80% confluence (3×10^5 cells) and transfected with 1 μg each of the sEGS-RNA vectors and the pNL4-3-luc plasmid. The cells were incubated for 3 days before the total cellular RNA was isolated. RNA samples were treated with DNase I according to the manufacturer's specifications. To quantify the level of HIV-1 RNA, amplifications of HIV-1 mRNA and the internal control message GAPDH were incorporated into the reaction mixtures for the RT and PCR steps. A sample of 1 μg of total RNA was used as the template with the vif, tat, and GAPDH primers (20 pmol each). Reverse transcription (final volume: 50 μl) was carried out at 60°C for 30 min. The cDNA products were amplified by PCR (94°C, 60 sec; 60°C, 90 sec; 40 cycles). The vif-F1 (5′-AGGAGAAAGAGACTGGCATTTGGG-3′) and tat-F1 (5′-ATGGAGCCAGTAGATCCTAGATCAGA-3′) primers and the vif-R (5′-CTCCTTCTGTCGAATAACGCCTATTCTG-3′) and tat-R (5′-CTATTCCTTCGGGCCTGTCGGGTC-3′) primers only generated a cDNA product from the uncleaved HIV-1 mRNA (RT-PCR product I, vif = 500 base

pairs, product II, tat = 200 base pairs), whereas the GAPDH-F and R primers generated the GAPDH gene (0.45 kb) as the internal control.

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Characterization of baculovirus *Autographa californica* multiple nuclear polyhedrosis virus infection in mammalian cells

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Received 20 February 2006
Available online 9 March 2006

Abstract

The baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is used as a vector in many gene therapy studies. Wild-type AcMNPV infects many mammalian cell types in vitro, but does not replicate. We investigated the dynamics of AcMNPV genomic DNA in infected mammalian cells and used flow cytometric analysis to demonstrate that recombinant baculovirus containing a cytomegalovirus immediate early promoter/enhancer with green fluorescent protein (GFP) expressed high levels of GFP in Huh-7 cells, but not B16, Raw264.7, or YAC-1 cells. The addition of butyrate, a deacetylase inhibitor, markedly enhanced the percentage of GFP-expressing Huh-7 and B16 cells, but not Raw264.7 and YAC-1 cells. The addition of 5-aza-2′-deoxycytidine, a DNA methylation inhibitor, had no enhancing effect. Polymerase chain reaction analysis using AcMNPV-*gp64*-specific primers indicated that AcMNPV infected not only Huh-7 and B16 cells, but also Raw264.7 and YAC-1 cells in vitro. The genomic DNA was detected in Huh-7 and B16 cells 96 h after infection. Genomic AcMNPV DNA in YAC-1 cells was not transported to the nucleus. Luciferase assay indicated that AcMNPV *p35* gene mRNA and p35 promoter activity were clearly expressed only in Huh-7 and B16 cells. These results suggest that viral genomic DNA expression is restricted by different host cell factors, such as degradation, deacetylation, and inhibition of nuclear transport, depending on the mammalian cell type.

Keywords: Baculovirus; Vector; *gp64*; *p35*; Mammalian cells

The ability of baculovirus to infect insects has many applications. In particular, *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is used in many protein expression systems as a viral vector and plant insecticide.

AcMNPV has a double-stranded circular DNA genome of approximately 130 kbp containing more than 150 open-reading frames [1]. AcMNPV produced from infected cells has two viral phenotypes, occlusion-derived virus (ODV) and budded virus (BV) [2]. ODV enters midgut cells, and BV buds from infected midgut cells establish systemic infection by infecting hemocytes and other tissues in the larval lepidopteron host [3]. The viral genomic DNA of both phenotypes transports to the nucleus of the infected cells and replicates using nine essential viral genes, *ie-1*, *ie-2*, *p143*, *dnapol*, *lef-1*, *lef-2*, *lef-3*, *pe38*, and *p35* [4]. Moreover, the host range of AcMNPV in insect cells is detected by measuring the expression of viral helicase *p143* and pan-caspase inhibitor *p35* [5–8]. Argaud et al. [9] reported that recombinant AcMNPV *p143* replaced with *Bombyx mori* nuclear polyhedrosis virus (BmNPV) could replicate in non-permissive Bm5 cells. Clem and Miller [8], using a *p35* mutant virus, demonstrated protection of viral-induced apoptosis by expression of the AcMNPV *p35* gene within the host range.

AcMNPV infects a variety of mammalian cell types in vitro, but not certain hematopoietic cell lines, by unknown

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.02.167

mechanisms. The inability of baculoviruses to replicate in mammalian cells makes them attractive candidate vectors for in vitro gene therapy studies [10,11]. These recombinant vectors contain compatible promoters and are highly effective in infecting primary hepatocyte and hepatoma cell lines, making them very useful tools for studies of hepatitis B and hepatitis C viruses [12–14].

To enhance gene delivery of the baculovirus vector into mammalian cells, a vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped baculovirus vector was generated to improve foreign gene expression in mammalian cells compared to wild-type baculovirus vector [15]. The VSV-G pseudotyped virus is thought to facilitate particle entry and escape from endolysosomes. Schauber et al. [16], however, reported that transduction of the baculovirus *gp64* pseudotyped lentiviral vector is similar to that of the VSV-G pseudotyped lentiviral vector, except for in hemapoietic cells. Furthermore, Tani et al. [17] suggested that baculovirus *gp64* vectors directly interact with general phospholipids in the surface membrane of mammalian cells, and the VSV-G pseudotyped virus interacts with general phosphatidylserine. Therefore, we hypothesized that baculovirus *gp64* mediates transduction in the presence of intracellular or viral factors.

In the present study, we investigated baculovirus infection and transduction in permissive and non-permissive mammalian cells in vitro. We report that AcMNPV penetrated not only Huh-7 cells, but also the hematopoietic cell lines, Raw264.7 and YAC-1 cells, while Raw264.7 and YAC-1 cells inhibited GFP gene expression. Nuclear transport of the AcMNPV genome was inhibited in YAC-1 cells. Our data suggest that AcMNPV might be useful for the development of more efficient baculovirus vectors for gene therapy.

Materials and methods

Animals and cell lines. Female C57BL/6 mice were purchased from Nippon SLC (Hamamatsu, Japan) and used at 6–8 weeks of age. A human hepatoma cell line (Huh-7), a mouse melanoma cell line (B16), a mouse lymphoma cell line (YAC-1), and a mouse macrophage cell line (Raw267.4) were obtained from the Riken Cell Bank (Wako, Japan). Huh-7, B16, and Raw264.7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St Louis, MO) and YAC-1 in RPMI 1640 (Sigma Chemical Co.), both supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Where indicated, culture medium was supplemented with 5-aza-2′-deoxycytidine (Aza-C; Sigma Chemical Co.) or sodium butyrate (Wako Chemical Co., Osaka, Japan) after virus treatment.

Generation of recombinant baculovirus. Wild-type baculovirus AcMNPV was purchased from BD Bioscience (San Diego, CA). The GFP was moved from pLEGFP-C1 (BD Bioscience) to pcDNA3.1(+) (Invitrogen Corp., Carlsbad, CA) as a *NheI-XhoI* fragment to construct pcDNA.GFP. Viruses were constructed using shuttle vectors derived from pVL1393 (BD Bioscience). The shuttle plasmid DNA was digested using a *SmaI-EcoRI* fragment. A 3.1 kbp BamHI-MunI fragment from pcDNA.GFP, which contains the CMV-IE promoter/enhancer with a GFP gene and polyadenylation signal expression cassette, was inserted into the pVL1393 backbone (pVL1393/CMV-GFP). Recombinant baculovirus AcCMV-GFP was generated according to the manufacturer's instructions (BD Bioscience). Purification of the baculovirus was performed as described previously [18].

Transduction of mammalian cells by baculovirus. Cells were seeded in 35 mm culture dishes at 3×10^5 cells per dish. Culture medium was removed, replaced with virus treatment, and incubated for 1 h at 37 °C. After removal of the virus, fresh medium was added and cultures were incubated at 37 °C. Cultures were harvested, washed, and resuspended in phosphate-buffered saline. The GFP-expressing populations were analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA).

Polymerase chain reaction. Total DNAs were extracted from cells using the GenEluteTM Mammalian Genomic DNA Miniprep Kit (Sigma Chemical Co.) according to the manufacturer's instructions. Locations of the baculovirus genome were determined as described previously [15]. One-step polymerase chain reaction (PCR) was performed on 50 ng DNA samples using KOD-Plus—(Toyobo, Osaka, Japan). The specific primer pairs are presented in Table 1.

Construction of plasmids and luciferase assay. The enhancer *hr5* and p35 promoter were cloned from the AcMNPV genome to a pCR2.1-TOPO vector (Invitrogen), and the sequence homology was verified. The sequences of the specific primers were as follows: hr5: 5′-GAGCTCTTGC ACAATGTAACTAGTGCACTC-3′ (sense) and 5′-GCTAGCCGTCCG TTTGATTTAACG-3′ (anti-sense); p35 promoter: 5′-CTCGAGGTCGG TCACCATGTACAAAAG-3′ (sense) and 5′-AGATCTGCTCAAATGC TCACTTAATACAAG-3′ (anti-sense). The pGL.hr5.p35prom vector was constructed by a 705-bp SacI-NheI fragment containing hr5 and a 503-bp XhoI-BglII fragment containing the p35 promoter in a firefly luciferase reporter pGL3-basic vector (Promega Corp., Madison, WI). Transfections were performed by using Fugene 6 (Roche Applied Science), according to the manufacturer's instructions. Cells were plated at $0.8–3.0\times10^5$ cells per well in 24-well plates 24 h before transfection. Reporter plasmid (400 ng) was mixed with 2 μl Fugene 6 in 50 μl serum-free medium, left for 15 min at room temperature, and then added to the cells. The pRL-TK vector (Promega) containing the herpes simplex virus thymidine kinase (HSV-TK) promoter driving the expression of a renilla luciferase reporter was used as an internal control for transfection efficiency (20 ng per transfection). Cells were harvested and lysed 24 h post-transfection. The firefly and renilla luciferase activities were measured using the Promega Dual luciferase assay system with 20 μl of cell extract according to the manufacturer's instructions with a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA).

Results and discussion

Recombinant baculovirus-mediated GFP gene expression in mammalian cells in vitro

Infectivity and intensity of expression of AcCMV, which uses the CMV-IE promoter to express the GFP gene, was

Table 1
Primers used for PCR[a]

Gene	Sense	Anti-sense	Annealing temp (°C)
gp64	CTACTAGTAAATCAGTCACACC	CCAAGTTTTTAATCTTGTACGG	50
p35	GGTAGAAATCGACGTGTCCCAGA	CGTGAGCAAACGGCACAATAAC	56
p143	TAATGTATCCAGGGTCGGTGCTCT	CGCATCATCATGTCCAAAGTGGAC	56
G3PDH	TCCACCACCCTGTTGCTGTA	ACCACAGTCCATGCCATCAC	60

[a] All sequences are presented in the 5′–3′ direction.

investigated by FACS analysis using the permissive cell lines Huh-7 and B16, and non-permissive cell lines Raw264.7 and YAC-1. These cells were infected with AcCMV-GFP (MOI 50) following the addition of a DNA methylation inhibitor, Aza-C, or a deacetylase inhibitor, butyrate, and the cells were harvested 24 h later. Condreay et al. and Liang et al. [19,20] reported that butyrate enhances the expression of recombinant baculovirus in mammalian cells, but the effects of DNA methylation inhibitior have not been reported. The percentage of GFP-expressing cells and the mean fluorescence intensity of AcCMV-GFP were detected by flow cytometric analysis. The percentage of GFP-expressing Huh-7 cells was 21.9%, higher than in the other cell lines (B16, Raw264.7, and YAC-1; Fig. 1A). Flow cytometric analysis did not detect GFP expression in Raw264.7 or YAC-1 cells. The mean fluorescence intensity of AcCMV-GFP-infected cell lines was higher in Huh-7 cells than in the other cell lines (Fig. 1B). Butyrate, but not Aza-C, increased the percentage of GFP-expressing cells approximately 2-fold in Huh-7 cells and 10-fold in B16 cells. Mean fluorescence intensity of GFP expression was enhanced approximately 3-fold in Huh-7 cells and 2-fold in B16 cells. Nusinzon and Horvat reported that a deacetylase inhibitor inhibits the anti-viral response of interferon-stimulated transcription [21]. Transduction of these cells was not markedly enhanced, however, when Aza-C was used as the DNA methylation inhibitor. Therefore, expression

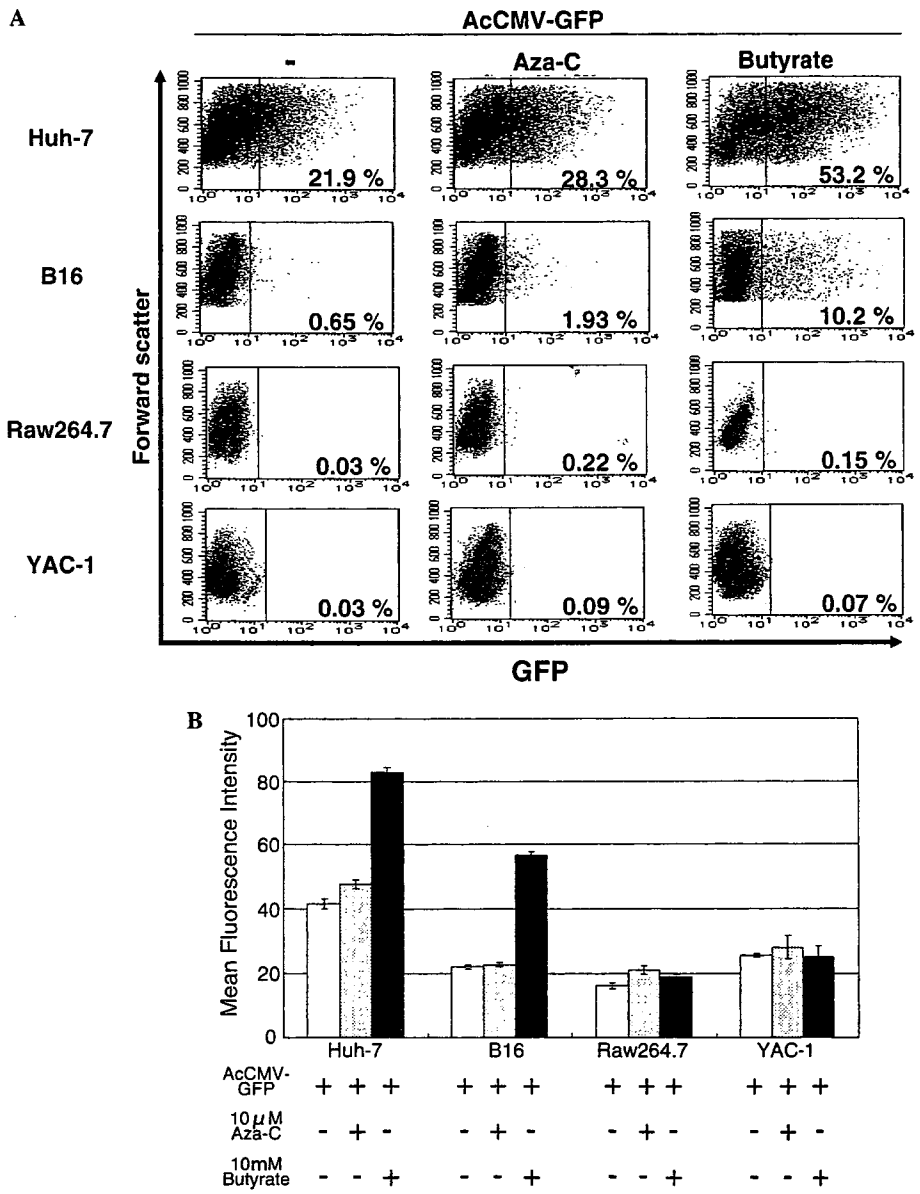

Fig. 1. In vitro recombinant baculovirus transduction in permissive or non-permissive cell lines. The cells were treated with recombinant baculovirus (AcCMV-GFP; MOI 50) or vehicle. After culturing in culture medium with or without 10 μM Aza-C or 10 mM butyrate for 24 h, harvested Huh-7, B16, Raw264.7, and YAC-1 cells were analyzed to determine the percentage of GFP-expressing cells (A) and mean fluorescence intensity of the GFP (B) using flow cytometry. The data shown are representative of duplicate experiments that gave similar results.

and transcription, but not infectivity, of recombinant baculovirus was enhanced in Huh-7 and B16 cells to which butyrate was added to induce hyperacetylation, but not in cells in which chromatin methylation was inhibited. Thus, we suggest that recombinant baculovirus can enter Huh-7 and B16 cells, although GFP expression of recombinant baculovirus was inhibited by acetylation in these cells due to cellular anti-viral function.

AcMNPV infects permissive and non-permissive cell lines

Barsoum et al. [15] reported that the VSV-G pseudotype baculovirus as well as wild-type baculovirus could be modified for more efficient transduction, because baculovirus infection produces a low level of viral DNA. We then determined whether the cell lines were infected with AcMNPV. The cells were treated with one of several MOIs (1, 10, 50, 100, or 200) of AcMNPV and harvested 24 h later. AcMNPV-infected cells were detected from total DNA of infected cells by PCR amplification using AcMNPV-*gp64*-specific primers. Surprisingly, AcMNPV-*gp64*-specific bands from total DNA of infected cells were detected not only in Huh-7 and B16 cells, but also in Raw264.7 and YAC-1 cells (Fig. 2). Furthermore, specific bands of the viral genome were also detected in mouse primary splenocytes (data not shown). The AcMNPV-specific bands from cells infected with AcMNPV (MOI 1) were detected only in Huh-7 cells. These findings suggest that baculovirus AcMNPV can enter various cell lines, even when the recombinant baculovirus containing the expression cassette is not expressed.

Degradation and nuclear transport of viral DNA in the infected cells

We investigated whether the genomic DNA is degraded in the infected cells to produce cellular resistance to the virus. Wang et al. [22] demonstrated that baculovirus DNA is degraded in hepatoma cells. Condreay et al. and Merrihew et al. [19,23], however, reported that genomic DNA of recombinant baculovirus expressing neomycin phosphotransferase is integrated into CHO cells at a rate of approximately 1 in 50–100 cells. To determine if the cells contain viral DNA, the cells were treated with AcMNPV (MOI 50) and harvested at various time points. Cells invaded by AcMNPV were detected by PCR amplification using AcMNPV-*gp64* specific primers under dividing conditions. Viral DNA bands were confirmed in Huh-7 and B16 cells up to 96 h after infection (Fig. 3A). In the non-permissive cell lines Raw264.7 and YAC-1, however, AcMNPV (MOI 50) survived for up to only 48 h after infection. These results suggest that in Huh-7 and B16 cells, baculovirus viral DNA is taken up more easily or the viral DNA is slower to degrade.

Cells were infected with AcMNPV, total DNA was extracted 24 h later, and nuclear and cytoplasmic DNA was isolated as described in Materials and methods section. In adherent Huh-7, B16, and Raw264.7 cells, the AcMNPV genome was transported to the nucleus (Fig. 3B). In contrast, in suspension cells (YAC-1), the genomic DNA was not transported to the nucleus. van Loo et al. [24] suggested that the baculovirus genome is transported by actin filaments in mammalian cells, as in insect cells. The CMV-IE promoter strongly enhances binding with RNA polymerase II in the nucleus [25]. Recently, Salminen et al. [26] reported that human hepatocytes block nuclear transport of the baculovirus genome by microtubules, in contrast to insect cells. These results suggest that the recombinant

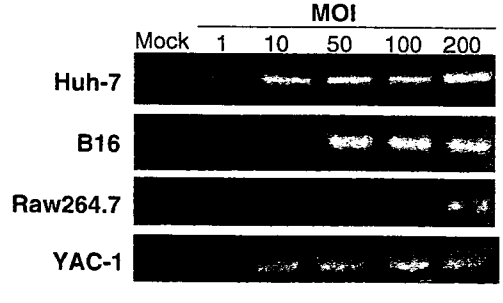

Fig. 2. AcMNPV MOI-dependent infection in various mammalian cells. The cells were treated with AcMNPV (MOIs 1, 10, 50, 100, and 200). The cells were harvested after 24 h. AcMNPV genomic DNA was detected in isolated total DNA of treated cells by PCR using AcMNPV-*gp64*-specific primers. The data shown are representative of duplicate experiments that gave similar results.

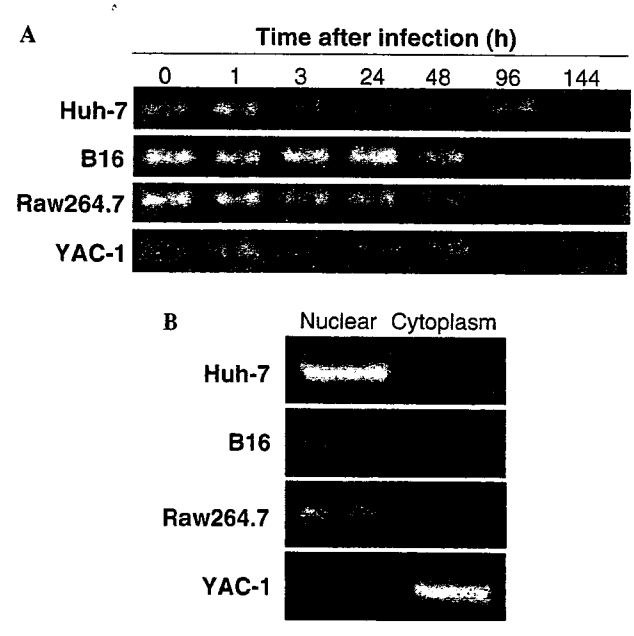

Fig. 3. Degradation and nuclear transport of the AcMNPV genome in various mammalian cells. (A) The cells were treated with AcMNPV (MOI 50). These cells were harvested at the indicated times, and total DNA was isolated. (B) The cells were treated with AcMNPV (MOI 50). The cells were harvested after 24 h, and the nuclear and cytoplasmic DNA was isolated as described in Materials and methods section. AcMNPV genomic DNA was detected in isolated DNA by PCR using AcMNPV-*gp64*-specific primers. The data shown are representative of duplicate experiments that gave similar results.

baculovirus did not activate the CMV-IE promoter in the infected macrophage cell line Raw264.7, and was inhibited by degradation of the viral genome and an unknown mechanism in infected YAC-1 due to inhibited nucleus transport of the genomic DNA into the nucleus.

AcMNPV expression determined using p35 gene detection in Huh-7 and B16 cells

The host range of AcMNPV in insect cells is involved in the expression of pan-caspase inhibitor p35 and viral helicase p143 [5–9]. We examined whether insect host range-detecting genes *p35* and *p143* were expressed in mammalian cells using reverse transcription RT-PCR analysis with *p35* or *p143*-specific primers. The cells were infected with AcMNPV and then harvested. Total RNA was extracted from infected cells 24 h later. The baculovirus *p35* gene was more clearly expressed in Huh-7 cells than in B16 cells, but was not expressed at all in the other cell lines (Fig. 4A). The AcMNPV *p143* gene, which is a host range-detecting gene in insect cells, was not expressed in mammalian cell lines.

To define the p35 promoter activity in mammalian cells, we constructed the luciferase reporter plasmid pGL.hr5.p35prom the inserted p35 promoter region and enhancer hr5 of the p35 promoter (Fig. 4B). For a transient reporter assay, cells were transfected with the reporter plasmid, and the reporter assay was examined 24 h later. Relative luciferase activity of baculovirus p35 promoter was clearly enhanced in Huh-7 and B16 cells. Based on the expression of *p35* mRNA, relative luciferase activity in Huh-7 cells was enhanced 2.4-fold compared to the activity in B16 cells. As expected, relative luciferase activity in host Sf9 cells was the highest in these cell lines (Table 2). These results suggest that the baculovirus p35 promoter was clearly activated in non-host mammalian cells, as in Huh-7 and B16 cells.

The AcMNPV *p35* gene has multiple functions. AcMNPV p35 inhibits apoptosis by inhibiting pan-caspase and mitigating eIF2α phosphorylation in various cell lines, C. elegans cells, drosophila cells, and mammalian cells [27–31]. Although the baculovirus BmNPV *p35* gene shares approximately 90% identity with the AcMNPV *p35* gene, BmNPV *p35* does not block apoptosis very well, compared

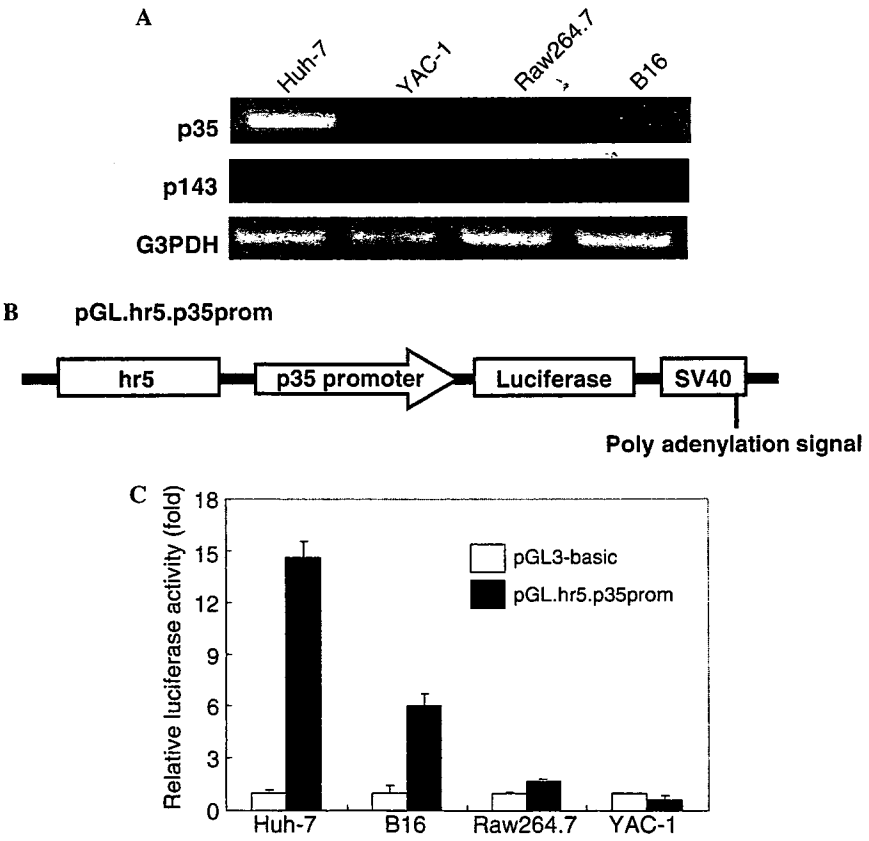

Fig. 4. Expression of AcMNPV p35 in various mammalian cells. (A) The cells were treated with AcMNPV (MOI 50). The cells were harvested after 24 h. The AcMNPV *p35* or *p143* gene was detected in isolated total RNA of treated cells by RT-PCR using specific primers. The data shown are representative of duplicate experiments that gave similar results. (B) Reporter plasmid containing the luciferase gene was placed under the control of the p35 promoter and enhancer hr5 was constructed. (C) The cells were co-transfected with pGL3-basic plasmid (no insert) or reporter plasmid (400 ng/well) and pRL-TK plasmid (20 ng/well) as an indicator for normalization of transfection efficiency. Cell extracts were prepared 24 h later and assayed for luciferase activity. Ratio of relative luciferase activity represents the firefly luciferase to renilla luciferase ratio with the pGL3-basic plasmid value defined as 1. The values reported represent averages of duplicate transfection.