VIRAL FACTORS INVOLVED IN ADAPTER-RELATED PROTEIN COMPLEX 2 ALPHA 1 SUBUNIT-MEDIATED REGULATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REPLICATION

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Abstract. The presence of siRNA against adapter-related protein complex 2 alpha 1 subunit (AP2α) enhances human immunodeficiency virus type 1 (HIV-1) replication by up-regulating nuclear transport of viral genome. In this report, we examined possible viral factors involved in AP2α-mediated regulation of HIV-1 replication, namely, Gag matrix protein (MA), integrase (IN) and Vpr. Replication of mutant viruses lacking the nucleophilic property of one of these viral proteins was significantly enhanced by treating cells with AP2α siRNA, indicating that Gag MA, IN or Vpr is not specifically involved in AP2α-mediated enhancement of viral replication. In contrast, AP2α siRNA showed no effect on the level of gene transduction mediated by HIV-1-derived lentiviral vector (LV). Although virus-like LV particle and parental HIV-1 particle are composed of almost equivalent viral structural proteins, LV particles lack three accessory proteins, Vif, Vpr and Vpu, and a large portion of the HIV-1 genome. Vif, Vpr and Vpu were dispensable for AP2α siRNA-mediated enhancement of HIV-1 replication, indicating that a particular part of the HIV-1 genomic fragment deleted in the LV genome might be required for the enhancing effect of AP2α siRNA on viral replication. Taken together, these results suggest that an as yet undetermined gene fragment of the HIV-1 genome is involved in AP2α-mediated regulation of HIV-1 replication.

Keywords: HIV-1 replication, viral factors, AP2α siRNA

INTRODUCTION

We previously had studied the level of human immunodeficiency virus type 1 (HIV-1) replication in cells transfected with a series of small interfering RNA (siRNA) directed against cellular genes selected as functional molecules involved in intracellular signal transduction pathways, intracellular transport processes and cytoskeletal system (siRNA mini-library) (Ui-Tei *et al*, 2004; Kameoka

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et al, 2007), and found that the levels of the single replication cycle of luciferase reporter HIV-1 were significantly enhanced in cells transfected with siRNA against adapter-related protein complex 2 (AP-2) alpha 1 subunit (AP2α) (Kameoka *et al*, 2007). These results suggested that AP2α negatively regulated HIV-1 replication under normal cell culture conditions. In addition, our subsequent study revealed that nuclear transport of the HIV-1 genome was negatively regulated by AP2α (Kitagawa *et al*, 2008).

AP2α is a major component of AP-2 that regulates receptor-mediated, clathrin-dependent endocytosis of plasma membrane proteins (Conner and Schmid, 2003; Nakatsu and Ohno, 2003); thus, the major population of AP2α is localized at the plasma membrane (Kitagawa *et al*, 2008). However, our previous results suggested that a subpolulation of AP2α is localized in the cytoplasm and the perinuclear region, and plays a role in suppressing nuclear translocation of HIV-1 genome (Kitagawa *et al*, 2008). Viral nucleophilic proteins, Gag matrix protein (MA), integrase (IN) and Vpr, are associated with HIV-1 intracellular reverse transcription complex (RTC) or preintegration complex (PIC), and individually play roles in the nuclear transport of the HIV-1 genome (Bukrinsky *et al*, 1993; Heinzinger *et al*, 1994; von Schwedler *et al*, 1994; Gallay *et al*, 1997; Bouyac-Bertoia *et al*, 2001).

In this report, we examined the involvement of these nucleophilic proteins in AP2α-mediated regulation of HIV-1 replication. In addition, we examined the possible involvement of AP2α in regulating the efficiency of gene transduction mediated by pNL4-3 (Adachi *et al*, 1986)-derived lentiviral vector (LV), in order to further study the role of AP2α in HIV-1 replication.

MATERIALS AND METHODS

Cells

293T and MAGIC5A [CCR5-expressing MAGI (Hela-CD4-LTR-β-gal)] (Mochizuki *et al*, 1999; Hachiya *et al*, 2001) were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (complete medium).

siRNA

siRNA against AP2α (AP2α siRNA) (Kameoka *et al*, 2007; Kitagawa *et al*, 2008), was purchased from Gene Design, (Osaka, Japan). siRNAs against Apaf-1 (Apaf-1 siRNA) (#1024594; Qiagen, Hilden, Germany) and the control (non-silencing) siRNA (control siRNA) (#1022076, Qiagen) were used as negative controls. Cells were transfected with siRNA (20 nM final) using HiPerfect transfection reagent (Qiagen), as described previously (Kitagawa *et al*, 2008).

Construction of envelope protein (Env)-deficient, luciferase reporter proviral constructs lacking nucleophilic property of HIV-1 Gag MA, Vpr or IN

pNL4-3-derived, envelope protein (Env)-deficient, luciferase reporter viral construct, pNL-Luc-E$^-$-gagpolCT (denoted as pNL-Luc) (Kitagawa *et al*, 2008) was used as wild-type HIV-1 in this study. The pNL-Luc-derived mutant virus lacking two nuclear localization signals (NLSs) of Gag MA was generated by site-directed mutagenesis using the QuikChange® site-directed mutagenesis kit (Stratagene, Cedar Creek, TX), according to previous report (Haffar *et al*, 2000). In brief, two NLSs of HIV-1 Gag MA were disrupted by introducing mutations, K26I [amino acid substitution from lysine (K) to isoleucine (I) at position 26], K27I, K112I and K113I into *gag* of pNL-Luc. In ad-

dition, the nucleophilic property of Vpr was removed by introducing a mutation, F34I, into *vpr* of pNL-Luc, according to the previous report (Vodicka *et al*, 1998). The mutant virus lacking IN was generated by introducing a stop codon at amino acid position 7 of *integrase* of pNL-Luc. Generated mutants were designated as pNL-MA K26I/K27I/K112I/K113I, pNL-Vpr F34I and pNL-IN$^-$.

Construction of Env-deficient, luciferase reporter proviral constructs lacking HIV-1 Vif-, Vpr- and/or Vpu

Vif (Kinomoto *et al*, 2007)-, Vpr (Tokunaga *et al*, 2001)- and Vpu (Iwabu *et al*, 2009)-deficient proviral constructs, denoted in this study as pNL-Luc-F$^-$, pNL-Luc-R$^-$ and pNL-Luc-U$^-$, were constructed by introducing frameshift mutations, which created premature stop codons, into *vif*, *vpr* and *vpu* of pNL4-3-derived, Env-deficient, luciferase reporter proviral construct equivalent to pNL-Luc, respectively. In addition, Vif / Vpr double-deficient proviral construct, pNL-Luc-F$^-$R$^-$, was constructed by introducing a frameshift mutation into *vif* of pNL-Luc-R$^-$, as described previously (Kinomoto *et al*, 2007). In addition, Vif /Vpu double-deficient proviral construct, pNL-Luc-F$^-$U$^-$, and Vpr /Vpu double-deficient proviral construct, pNL-Luc-R$^-$U$^-$, were constructed by replacing the *Sal*I-*Not*I fragment of pNL-Luc-F$^-$ and pNL-Luc-R$^-$, respectively, with that of pNL-Luc-U$^-$. Finally, Vif /Vpr /Vpu triple-deficient proviral construct, pNL-Luc-F$^-$R$^-$U$^-$, was constructed by replacing the *Sal*I-*Not*I fragment of pNL-Luc-F$^-$R$^-$ with that of pNL-Luc-U$^-$.

pNL4-3-derived LV and packaging constructs

Self-inactivating LV construct, pWPT-GFP (Zufferey *et al*, 1998), the second-generation packaging construct, pCMVΔR8.91 (Zufferey *et al*, 1997), and the vesicular stomatitis virus G protein (VSVG) expression vector, pMD2.G (Zufferey *et al*, 1997), were kindly provided by Dr Didier Trono, School of Life Sciences and "Frontiers in Genetics", National Center for Competence in Research, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland. In order to generate LV construct containing *luciferase*, pWPT-Luc, the gene fragment encoding green fluorescent protein (GFP) of pWPT-GFP was replaced with *luciferase* derived from pNL-Luc.

Preparation of recombinant virus and LV particles

Recombinant virus was prepared by co-transfecting 293T cells with Env-deficient proviral construct and VSVG-expression vector, pHIT/G (Fouchier *et al*, 1997), using FuGENE HD transfection reagent (Roche, Basel, Switzerland), as described previously (Kitagawa *et al*, 2008). In addition, virus-like LV particles were prepared by co-transfecting 293T cells with pWPT-Luc, pCMVΔR8.91 and pMD2.G, essentially as described previously (Zufferey *et al*, 1998). Forty-eight hours after transfection, cell culture supernatants were centrifuged at 1,220 rpm for 15 minutes and stored as aliquots at -85ºC. Viral titer was determined by measuring the concentration of HIV-1 Gag p24 antigen in viral supernatants by enzyme-linked immunosorbent assay (ELISA) (BioAcademia, Osaka, Japan).

Viral infection

MAGIC5A cells were incubated with cell culture supernatants containing recombinant virus or LV particles (30 ng of p24) for 1 hour at 37ºC. Viral fluid then was replaced with complete medium, and the cells were cultured for 24 hours at 37ºC. Samples then were lysed in 100 μl

of lysis buffer (Glo Lysis Buffer; Promega, Madison, WI), and luciferase activity in the lysate was determined using Steady-Glo luciferase assay system (Promega) with LB960 microplate luminometer (Berthold, Bad Wildbad, Germany). Alternatively, cellular DNA was extracted for real-time PCR analysis using QIAamp DNA blood mini kit (Qiagen, Hildren, Germany).

Evaluation of viral nuclear translocation

The extent of viral nuclear translocation was evaluated by measuring the level of 2-LTR circular form of viral DNA using inverted PCR, as described previously (Kitagawa *et al*, 2008). Real-time nested PCR was carried out as follows. Cellular DNA was subjected to the first PCR using Blend Taq^{plus} (Toyobo, Osaka, Japan) and a pair of primers, U3S (5'-GAGCCCTCAGATGCTGCATATAAG-3', nt 9484 to 9507) and U3AS (5'-GCAGCTCTCGGGCCATGTGACG -3', nt 306 to 285). The PCR conditions were 94ºC for 2 minutes, followed by 30 cycles at 94ºC for 30 secends, 58ºC for 30 secends and 72ºC for 30 secends, with a final extension step at 72ºC for 5 minutes. Then, a 1 µl aliquot of first PCR product was mixed with a 15 µl reaction mixture containing 2x TaqMan universal PCR master mix (Applied Biosystems, Carlsbad, CA), RS (5'-AACTAGGGAACCCACTGCTTAAG-3', nt 9575 to 9597) (300 nM) and U3AS2 (5'-TCCACAGATCAAGGATATCTTGTC-3', nt'51 to 28) (300 nM) and RU5 probe (5'-FAM-ACACTACTTGAAGCACTCAAGGCAAGCTTT-TAMRA-3', nt 9634 to 9605) (200 nM). Real-time PCR was carried out using ABI PRISM 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA), according to the manufacturer's instructions. The PCR product (2-LTR fragment), amplified by using the primer pair RS-U3AS2, was cloned in Bluescript II SK(+). The plasmid containing the 2-LTR fragment and cellular DNA of MAGIC5A cells then were serially diluted and subjected to real-time PCR to make standard plots, and the copy numbers of the 2-LTR circular form of HIV-1 DNA and cellular β-actin DNA were quantified by absolute quantification method. Then, the level of HIV-1 DNA was standardized by dividing the copy number of HIV-1 DNA by that of β-actin gene, and the results are presented as the relative amount of the 2-LTR form of HIV-1 DNA.

Immunoblotting

MAGIC5A cells were lysed in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecylsulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol and 0.003% bromophenol blue), and samples then were separated by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting. Protein blots were immunostained with anti-AP2α (adaptin α) monoclonal antibody (#610502; BD Biosciences, San Jose, CA) or anti-β-actin monoclonal antibody (A5316; Sigma-Aldrich, St Louis, MO), and with peroxidase-labeled secondary antibodies. The immuno-complex was visualized using ECL Western blotting detection reagent kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

RESULTS

Effects of Gag MA, Vpr and IN on AP2α-mediated regulation of HIV-1 replication

Our previous study revealed that AP2α siRNA enhances HIV-1 replication by up-regulating nuclear transport of the viral genome (Kitagawa *et al*, 2008). Nuclear translocation of HIV-1 genome is mediated by three nucleophilic viral proteins, Gag MA, IN and Vpr (Bukrinsky *et al*, 1993; Heinzinger *et al*, 1994; von

Schwedler *et al*, 1994; Gallay *et al*, 1997; Bouyac-Bertoia *et al*, 2001); therefore, we examined which nucleophilic viral proteins was involved in the AP2α-mediated regulation of HIV-1 replication using pNL-Luc-derived recombinant viruses containing NLS mutant of Gag MA and non-nucleophilic mutant of Vpr as well as the recombinant virus lacking IN. Recombinant virus generated from the reporter proviral construct, pNL-Luc, contains the *luciferase* reporter gene in place of viral *nef* gene; therefore, the efficiency of early phases of the HIV-1 replication cycle, including reverse transcription, integration, RNA transcription and protein translation, can be monitored by measuring luciferase activity in infected cells.

First, the cells were transfected with AP2α siRNA or control siRNAs. AP2α siRNA effectively suppressed the expression of AP2α in MAGIC5A cells (Fig 1). Cells then were infected with the recombinant viruses. The results showed that the replications of the recombinant viruses generated from the proviral constructs, pNL-MA K26I/K27I/K112I/K113I (MA NLS mutant) and pNL-Vpr F34I (non-nucleophilic Vpr mutant), were significantly enhanced by AP2α siRNA (Fig 2, left panel). In contrast, the luciferase activity expressed in infected cells by the recombinant viruses generated from pNL-IN$^-$ (IN mutant) was quite low (data not shown); therefore, it was impossible to evaluate the effect of AP2α siRNA on the replication of IN mutant virus by luciferase expression in infected cells. Thus, we evaluated the nuclear transport of the proviral DNA by real-time PCR. The results showed that the nuclear transport of proviral DNA of IN mutant virus, as well as of wild-type virus (data not shown), is significantly enhanced by treating cells with AP2α siRNA (Fig 2, right panel).

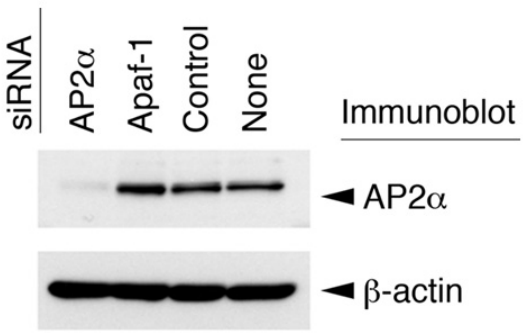

Fig 1–AP2α siRNA suppression of AP2α expression in MAGIC5A cells. MAGIC5A cells were mock-transfected (None) or transfected with AP2α siRNA, Apaf-1 siRNA or Control siRNA, as indicated above the panels. Forty-eight hours after transfection, cell lysates were prepared and subjected to immunoblot analysis using anti-AP2α (upper panel) or anti-β-Actin monoclonal antibody (lower panel).

These results suggest that the NLSs of Gag MA and the nucleophilic property of Vpr or IN were not specifically involved in the AP2α-mediated enhancement of HIV-1 replication.

Effect of AP2α siRNA on infection of virus-like LV particles

HIV-1-derived LV is able to efficiently transduce gene(s) into dividing and non-dividing cells (Naldini *et al*, 1996; Zufferey *et al*, 1997). We therefore examined whether AP2α siRNA enhanced gene transduction mediated by HIV-1 pNL4-3-derived LV. Cells were transfected with AP2α siRNA and then infected with the virus-like LV particles carrying *luciferase* reporter gene. The results showed that AP2α siRNA did not enhance the infection of LV particles, while it significantly enhanced the infection of the wild-type virus generated from pNL-Luc (Wild-type) (Fig 3). LV particles and the wild-type virus were composed

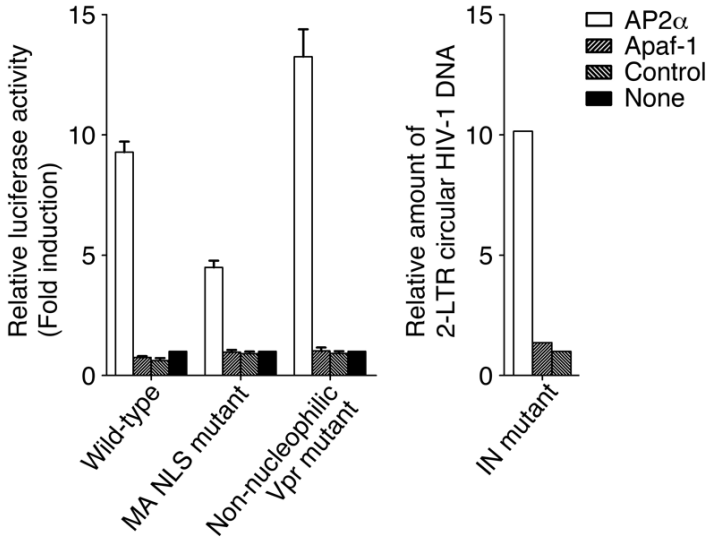

Fig 2–Effect on AP2α siRNA-mediated enhancement of HIV-1 replication by NLSs of Gag MA and integrase. MAGIC5A cells were mock-transfected (None, solid bar) or transfected with AP2α siRNA (open bar), Apaf-1 siRNA (left hatched bar) or Control siRNA (right hatched bar). Forty-eight hours after transfection, cells were infected with recombinant viruses generated from the proviral construct, pNL-Luc (Wild-type), pNL-MA K26I/K27I/K112I/K113I (MA NLS mutant), pNL-Vpr F34I (Non-nucleophilic Vpr mutant) or pNL-IN$^-$ (IN mutant), as indicated below the panel. Twenty-four hours after infection, luciferase activities in Wild-type-, MA NLS mutant- and Non-nucleophilic Vpr mutant-infected cells were measured. Data are shown as fold inductions relative to luciferase activity in mock-transfected cells. Data are presented as mean ± SE (error bar) of at least three independent experiments (left panel). In addition, twenty-four hours after infection, total cellular DNA of IN mutant-infected cells was extracted, and the extent of viral nuclear translocation was evaluated by real-time PCR, as described in Materials and Methods. Data are presented as mean of three independent experiments (right panel).

of almost equivalent viral structural proteins; however, the proviral construct, pNL-Luc, contains three HIV-1 accessory genes encoding Vif, Vpr and Vpu, whereas the packaging construct used to generate the LV particles, pCMVΔR8.91, does not contain these genes. In addition, a large proportion of the pNL4-3 genome was deleted from the LV construct, pWPT-Luc. Therefore, we next examined the possible role of these accessory gene products in the enhancing effect of AP2α siRNA on viral replication. For this purpose, we prepared pNL-Luc-derived accessory gene mutants lacking Vif, Vpr and/or Vpu, and examined the replication of these mutants in AP2α siRNA-transfected cells. The results showed that the replications of single (Vif mut, Vpr mut or Vpu mut), double (Vif, Vpr mut; Vif, Vpu mut; or Vpr, Vpu mut) and triple (Vif, Vpr, Vpu mut) accessory gene mutants were significantly enhanced by AP2α siRNA, similar to the wild-type virus (Fig 3). These results suggest that Vif, Vpr and Vpu were dispensable for the AP2α-mediated regulation of HIV-1 replication, and also implies the possibility that HIV-1 genomic fragment deleted from the LV construct is required for AP2α siRNA-dependent enhancement of viral replication.

Therefore, we tried to determine the genomic fragment of pNL-Luc involved in AP2α-mediated regulation of HIV-1 replication. The LV construct does not contain a large portion of the HIV-1 genome, including HIV-1 promoter region, coding regions for most HIV-1 proteins and un-

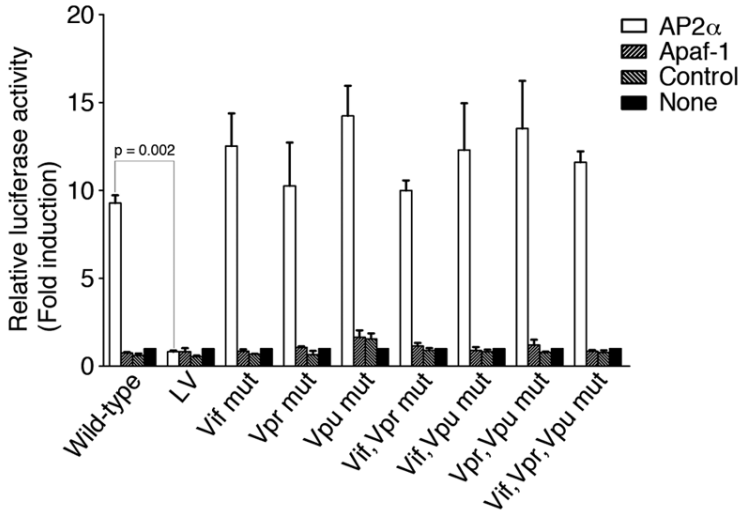

Fig 3–Effect of AP2α siRNA on infection of virus-like VL particles, and on replication of recombinant viruses generated from pNL-Luc and pNL-Luc-derived accessory gene mutants. MAGIC5A cells were mock-transfected (None, solid bar) or transfected with AP2α siRNA (open bar), Apaf-1 siRNA (left hatched bar) or Control siRNA (right hatched bar). Forty-eight hours after transfection, cells were infected with virus-like LV particles (LV) or recombinant viruses generated from the proviral construct, pNL-Luc (Wild-type), pNL-Luc-F$^-$ (Vif mut), pNL-Luc-R$^-$ (Vpr mut), pNL-Luc-U$^-$ (Vpu mut), pNL-Luc-F$^-$R$^-$ (Vif, Vpr mut), pNL-Luc-F$^-$U$^-$ (Vif, Vpu mut), pNL-Luc-R$^-$U$^-$ (Vpr, Vpu mut) or pNL-Luc-F$^-$R$^-$U$^-$ (Vif, Vpr, Vpu mut), as indicated below the panel. Twenty-four hours after infection, luciferase activity in infected cells was measured. Data are shown as fold induction relative to luciferase activity in mock-transfected cells. Data are presented as mean ± SE (error bar) of at least three independent experiments. Significant differences in the level of AP2α siRNA-mediated enhancement of viral replication among the recombinant viruses were analyzed by paired *t* test, and are reported when $p < 0.05$.

translated regions. A total of 7,408 of 9,709 base pairs of the HIV-1 genome, namely, nucletide positions 1142-7613, 8468-9004 and 9111-9509, are missing compared to parental pNL4-3. We attempted to identify the viral gene fragment required for the enhancing effect of AP2α siRNA on HIV-1 replication, but could not determine such regions.

DISCUSSION

Previous reports have shown that the contribution of three nucleophilic viral proteins to nuclear transport of the HIV-1 genome differs depending on the cellular context. Namely, NLS of integrase is sufficient for viral replication in dividing cells (Gallay *et al*, 1997), while the NLSs of Gag MA and the nucleophilic property of Vpr are required for viral replication in non-dividing cells (Bukrinsky *et al*, 1993; Heinzinger *et al*, 1994; von Schwedler *et al*, 1994). In addition, Gag MA and IN mediate nuclear transport of the HIV-1 genome through a classical nuclear transport pathway involving the interaction between importin α/β and these viral proteins (Gallay *et al*, 1996, 1997). In contrast, Vpr does not contain conventional NLS, but plays a role in the tethering of HIV-1 RTC or PIC to the nuclear pore complex (Jenkins *et al*, 1998; Vodicka *et al*, 1998). Our results suggest that AP2α might not be involved specifically in regulating nuclear transport pathway mediated by Gag MA, IN or Vpr, but it may be involved in upstream or downstream event of nuclear transport pathways of HIV-1 proviral DNA.

In addition, our study using pNL4-3-derived LV revealed that an as yet undetermined HIV-1 genomic fragment

is required for AP2α-mediated regulation of HIV-1 replication. Although we failed to determine the HIV-1 genomic region which affects the enhancing effect of AP2α siRNA on viral replication, we believe that these results provide important information for further examination of the regulatory mechanism of AP2α on HIV-1 replication.

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