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# SUPPRESSION OF ENDOGENOUS *hRIP* GENE EXPRESSION BY miRNA IN HELA AND 293T CELLS

Uraiwan Intamaso[1*], Panjit Chaiyasanit[1], Marut Tangwattanachuleeporn[1], and Somsak Pantuwatana[1]

[1]*Department of Medical Sciences, Faculty of Science, Burapha University, Chonburi 20131, Thailand.*

## ABSTRACT

A vector that expresses micro-RNA (miRNA) was designed and developed to regulate the cellular gene expression. The objective was aimed at the development of ribonucleic acid interference (RNAi) that could inhibit an expression of human Rev-interacting protein (hRIP). The hRIP has been shown to be an essential Rev cofactor that promotes the export of Rev-directed HIV RNA to the cytoplasm. We found that miRNA moderately suppressed hRIP-mRNA as detected by RT-PCR. This was in agreement with the reduction of hRIP protein as detected by Western blot. Stable miRNA expression was detected up to 48 h after transfection, possibly due to a cytotoxic side effect caused by transfection. If the efficiency of transfection can be improved and the cytotoxic effects minimized, an RNAi-based antiviral therapy might be used to interfere the HIV life-cycle.

**Keywords:** RNA interference hRIP, miRNA, Rev protein.

## INTRODUCTION

RNA interference is a sequence-specific gene silencing mechanism that is evolutionarily conserved in eukaryotes. It is considered to act as a natural defense against invading viruses and other infectious agents (Fire et al., 1998; Ketting et al., 1999; Li et al., 2002) as well as playing a role in post-transcriptional regulation of cellular gene expression (Szweykowska-Klinska et al., 2003; Voinet, 2002). It has been shown that small non-coding RNA, i.e., short interfering RNA (siRNA) and miRNA can mediate the gene silencing mechanism. The siRNA is short RNA-duplexes that guide the destruction of the perfectly complementary section of the mRNA through the RNAi pathway (Tuschl, 2002; Zamore, 2001). The miRNA can function through a mechanism that similar to siRNA. However, miRNA can bind only to partially complementary 3′ untranslated regions of mRNA directing a translational repression without target degradation (Lau et al., 2001; McManus et al., 2002; Lagos-Quintana et al., 2001; Lee et al., 2002). Most works have been concentrated on introducing synthetic siRNA into cultured cells. Those synthetic siRNA can cause a transient inhibition of homologous gene expression, which probably due to the dilution accompanied with cell division (Elbashir et al., 2001;

* Corresponding author. E-mail address: uraiwani@buu.ac.th

Holen et al., 2002). To overcome the shortcomings of chemically synthesized siRNA, several investigators have developed a DNA vector-mediated mechanism to generate short hairpin RNA (shRNA) that are converted into siRNA *in vitro* (Bummelkamp et al., 2002; Miyagishi and Taira, 2002) to provide a sustained effect. The RNAi that were made like miRNA were subsequently designed based on the observation that miRNA processed from a pre-miRNA precursor are more potent in inhibiting homologous gene expression than those expressed as conventional siRNA (McManus et al., 2002; Zeng and Cullen, 2003). Interestingly, when an endogenous miRNA was redesigned to be fully complementary to a target of choice, it mediated degradation of the target. It has been shown that robust levels of antisense RNA emerging from the shRNA expression system can cause sequence-specific toxicity in stratum of mice. However, this toxicity was attenuated when sequences that were toxic were placed into the artificial miRNA system without compromising target gene silencing efficacy (McBride et al., 2008). Moreover, the designing of an effector of RNAi in the context of a naturally occurring RNA polymerase (pol) II–driven miRNA transcript, also increases the flexibility of this approach allowing for conditional and cell type-specific expression (Stegmeier et al., 2005; Zhou et al., 2005; Shin et al., 2006).

The ability of RNAi to provide relative ablation of gene expression has opened up the possibility of using this as an antiviral infection mechanism by targeting viral genes required in the virus life-cycle. Several reports on the RNAi-mediated inhibition of viral infection in human cells have been documented, i.e., polio virus (Gitlin et al., 2002), hepatitis C (Kapadia et al., 2003; Wilson et al., 2003), influenza A virus (Ge et al., 2003), papilloma virus type 16 (Jiang and Milner, 2002), hepatitis B virus (Hamasaki et al., 2003), and human immunodeficiency virus-1 (HIV-1) (Lee and Rossi, 2004; Brisibe et al., 2003). However, it has been shown that RNAi worked best when directed against the highly conserved part of the viral gene. In the case of HIV-1 with a given high mutation rate during replication, RNAi therapy may be inactivated by escape mutations in the target portion of the viral genome (Boden et al., 2003). Thus, host cellular genes that can interrupt the HIV-1 life-cycle may offer particularly attractive targets because endogenous genes are not under immune pressure to generate escape mutants.

A cellular cofactor assisted Rev in cytosolic delivery of partially spliced and non-spliced HIV-1 mRNAs in the late stage of viral life-cycle (Malim et al., 1989; Hope, 1999; Cullen, 2002) was known and described in 1995 as the human Rev-interacting protein (hRIP) (Fritz et al., 1995). Bogerd and co-workers (Bogerd et al., 1995) also coiled the human Rev-interacting protein (hRIP) that was described by Fritz and co-workers as Rev-activation domain binding protein (Rab). In the absence of functional hRIP, Rev-directed RNA, mislocalized and aberrantly accumulated at the nuclear periphery, where hRIP localized, thereby prohibiting translation of viral mRNAs for the structural proteins of the virions in the cytoplasm (Sanchez-Velar et al., 2004). Another report further showed that the depletion of hRIP by a dominant-negative mutant or synthetic siRNA resulted in the loss of viral replication in human cell lines and primary macrophages (Yu et al., 2005). These results indicate that hRIP is an essential cellular factor for Rev function and HIV-1 replication.

In this study, an alternative model for vector-based expression of miRNA was pursued. Gateway-adapted expression vector (Invitrogen, USA) was chosen to drive the expression of engineered pre-miRNA sequences modified from an endogenous murine miR-155 sequence (Lagos-Quintana et al., 2001). The plasmids have expressed stem loop pre-miRNA in the presence of Pol II human cytomegalovirus (CMV) promoter, which were analogous to endogenous pre-miRNA with co-cistronic expression of Emerald green Fluorescence Protein (EmGFP) at the 5′ end. Once the expression processed by Dicer enzyme, a 22 nucleotide mature miRNA designed to be complementary to a section of *hRIP* mRNA guided the cleavage of the target. It

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was shown that miRNA specifically suppressed the expression of endogenous *hRIP*. Thus, the miRNA strategy might prove generally useful in reducing expression of target genes to provide the extended application of RNAi in viral-based therapy and to basic research including inhibiting novel gene expression to define gene function.

## MATERIALS AND METHODS

### Construction of vectors

The 22-nucleotide long miR sequence corresponding to *hRIP* mRNA nucleotide positions 757-777 or 1504-1524 was processed from a hairpin pre-miRNA expression vector. To generate the pre-miR *hRIP* constructs, oligonucleotides were designed by using Invitrogen's RNAi designer based on *hRIP* mRNA nucleotide sequence (GenBank accession number X89478), as shown in Table 1.

**Table 1.** Primers used in the construction of vectors.

| Name of primers | DNA sequence |
|---|---|
| HRIP 757-top | 5′-TGCTGATTTGCAGAATTCTGAGCTGC**GTTTTGGCCACTGACTGA**CGCAGC TCAATTCTGCAAAT-3′ |
| HRIP757-bottom | 5′-CCTGATTTGCAGAATTGAGCTGCGTCAGTCAGTGGCCAAAACGCAGCTCAGAATTCTGAAATC-3′ |
| HRIP 1504-top | 5′-TGCTGAAAGCTGGTGGGAAGACTGTA**GTTTTGGCCACTGACTGAC**TACAGTCTCCACCAGCTTT-3′ |
| HRIP1504-bottom | 5′-CCTGAAAGCTGGTGGAGACTGTAGTCAGTCAGTGGCCAAAA CTACAGTCTTCCCACCAGCTTTC-3′ |
| LacZ-top | 5′-TGCTGAAATCGCTGATTTGTGTAGTC**GTTTTGGCCACTGACTGA**CGACTACACATCAGCGATTT-3′ |
| LacZ-bottom | 5′-CCTGAAATCGCTGATGTGTAGTCGTCAGTCAGTGGCCAAAACGACTACACAAATCAGCGATTT C-3′ |

Mature miRNA sequences are underlined and sequences derived from miR-155 are in bold font.

The top and bottom primers were annealed to each other in equal amount of molar in an annealing buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.1 M NaCl) by heating at 95°C for 4 min followed by cooling to room temperature for 5-10 min. The formation of dsDNA was confirmed by its migration on a 4% agarose gel in TBE buffer prior to insertion into a linealized pcDNA™ 6.2-GW/EmGFP-miR (Invitrogen, U.S.A.) plasmid. This plasmid contains Pol II human CMV promoter and EmGFP immediately upstream from the inserted site. The addition of EmGFP in the pre-miR transcripts allowed tracking of miRNA expression. The constructed pmiR/*LacZ* was generated by annealing oligonucleotides provided with the Block-iT Pol II miR RNAi expression vector kits (Invitrogen, USA) in a similar fashion to use as a positive control vector for the inhibition study. In addition, the pcDNA™ 6.2-GW/EmGFP-miR plasmid vector that contains an insert was used as a negative control vector. The pcDNA™ 6.2-GW/EmGFP-miR plasmid vector that contains an insert was able to form a hairpin pre-miRNA but was predicted not to target any vertebrate gene. The ligated vectors were transformed into *E. coli* DH5α and transformants that were grown in low salt LB agar in the presence of spectinomycin at the concentration of 50 μg/ml were selected. Recombinant plasmids were extracted from *E. coli* DH5α cells and confirmed by sequencing prior to transfection. The recombinant plasmids were designated as pmiR/*hRIP*757 and pmiR/*hRIP*1504 for the constructed plasmids expressing pre-miRNA that specifically targeted *hRIP* mRNA bp 757-777 and 1504-1524, respectively. The pmiR/*lacZ* plasmid that targeted β-galactosidase (β-gal) mRNA was used as the positive control and pmiR vector was used as the negative control.

**Cell culture and DNA transfection**

Either HeLa or 293T cells were propagated in the complete RPMI 1640 (Gibco, USA) and DMEM media (Gibco, USA), respectively, at 37°C in a 5% $CO_2$ incubator. The growth medium was consisting of 10% fetal bovine serum (FBS) (Invitrogen, USA) supplemented with antibiotics, penicillin G and gentamycin, at the final concentrations of 100 and 0.10 µg/ml, respectively. In preparing the cell monolayers in 35 mm wells, a cell suspension containing $1.3x10^5$ cells in an incomplete appropriate medium without FBS and antibiotics was dispensed in a volume of 2 ml per well and incubated for 24 h prior to transfection. A 10 mm cover slip was placed at the bottom of the well prior to the dispersion of cells. Once the 50% confluent growth of cell monolayers were formed, they were transfected with 5 µg of expression vector in the presence of 5 µl lipofectamine 2000 (Invitrogen, USA) or in the ratio of 1:1 in a 35 mm well. The efficiency of transfection was determined by transfection of 250-pmol fluorescein-labeled dsRNA oligo (Invitrogen, USA). In addition, the transfection reaction was carried out with 3 µg of pmiR/*lacZ* and 500 ng of pcDNA™ 1.2/v5 of GW/*lacZ* reporter plasmid (or called *lacZ* reporter plasmid) (Invitrogen, USA) for a study of gene silencing. The incomplete media were replaced with fresh media supplemented with 10% FBS without antibiotics at 6 h post-transfection. The transfected cells were further incubated at 24, 48, and 72 h post-transfection for a time course experiment. At a given time interval, the cover slips that containing transfected cells on the surface were mounted with the Prolong® Gold antifade reagent (Invitrogen, USA) preparation to examine under a fluorescence microscope, and the rest of the transfected cells were harvested and used in other assays.

**RNA isolation and RT-PCR assay**

Total RNAs were isolated from either transfected HeLa cells or 293T cells with illustraRNAspin Mini Kit (Amersham Biosciences, UK) according to the manufacturer's protocol. One-step RT-PCR reaction was performed in a volume of 25 µl. The extracted RNA in the amount of 50 ng was added into 12.5 µl of master Mix 2X of FideliTag™ RT-PCR (Amers ham Biosciences, UK) in the presence of 1mM $MgCl_2$ and used for PCR-amplified using approximately 0.8 µM each of *hRIP*-specific sense primer (5´- AGACACAG CCTG CTTCATCA-3´) and antisense primer (5´- TCTGGCATTGGTCTGA AATG -3´) designed by software Program 3. The RT-PCR reaction was carried out according to manufacturer's protocol, generating 125 bp of RT-PCR product that was verified on 1% agarose gel electrophoresis that stained with SyberGold™ (Invitrogen, USA). The RT-PCR reaction was also performed with the GADPH-specific sense (5´-CAA CTACATGGTTTACATGTTC-3´) and antisense primers (5´-GCCAGTGGACTCCACGAC-3´) in order to normalize the samples for absolute RNA amount. These two primers were obtained from GenBank accession number E14134 and E14135, respectively.

**Protein extraction and Western blot assay**

Protein extracts were done by washing harvested cells with phosphate-buffered saline and lysed with the triple detergent lysis buffer containing proteinase inhibitor cocktail (Sigma-Aldrich, U.S.A.) in the ratio of $1x10^6$ cells per 50 µl lysis buffer. The triple detergent lysis buffer was consisting of 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP40, and 0.5% Sodium deoxycholate. The sample was mixed periodically using vortex for 1-2 h on ice, and lysates were centrifuged at 12,000 rpm for 15 min at 4°C to remove cell debris. The concentrations of protein were determined with protein assay kits (BioRad, U.S.A.). Twenty micrograms of crude extracts were resolved on 12.5% SDS-polyacrylamide gels and transferred onto polyvinulidene difluoride (PVDF) membrane. The samples on the membrane were sequential probed, and blots were stripped in a buffer (100 mM, 2-Mercaptoethanol, 2%SDS, 62.5 mM Tris-HCl pH 6.7) at 55°C for 30 min. One of either rabbit anti β-gal IgG at the concentration of

1:5000 (Zymed, U.S.A.) or goat anti-RIP/Rab (C-19) IgG at the concentration of 1:200 (Santacruz, U.S.A.) or rabbit anti β-actin IgG at the concentration of 1:200 (Sigma Aldrich, U.S.A.) was used to detect β-galactosidase, hRIP, and β-actin protein, respecti vely. Immuno-detection was performed using the peroxidase-based ECL detection system (Amersham BioScience, UK) according to the manufacturer's instructions.

## RESULTS

**Expression of pre-miRNA expression vector in cultured cells**

GFP-positive HeLa cells transfected with pmiR/*lacZ* were first detected at 24 h and increased to a maximum level at 48 h, as shown in Figure 1A, whereas the EmGFP expression at 72 h after transfection was almost unrecognizable. The expressions in cells transfected with pmiR/*hRIP*1504 and pmiR, as shown in Figures 1B and 1C, were similar to those found in 293T cells (data not shown). Overall transfection efficiencies of the expression vectors at 48 h were approximately 50-60% for both cell lines. In contrast, almost 100% of EmGFP expressed cells were observed only at 24 h after transfection when transfected with oligo-fluorescense with a characteristic spotty distribution, as shown in Figure 1D. These results indicated that cells had taken up oligonucleotides more efficiently than expression vectors, but the oligonucleotides were less stable.

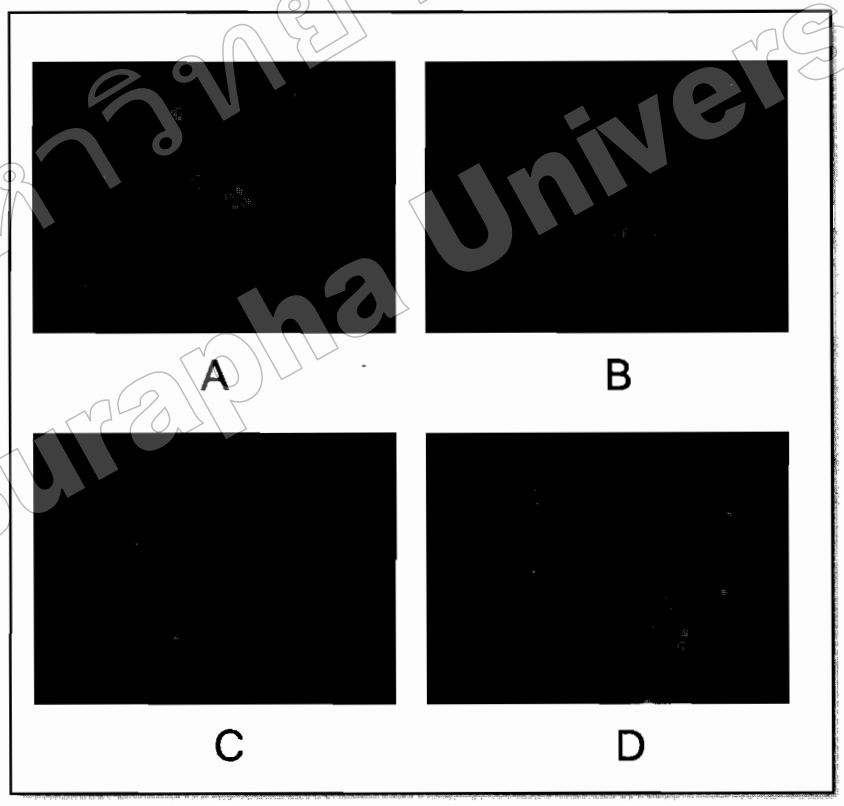


**Figure 1.** Fluorescence photographs of GFP-positive cells 48 h after transfection of HeLa cells with pmiR/*lacZ* (A), pmiR/*hRIP* 1504 (B), and pmiR (C), whereas that with fluorescein labeled RNA oligo (D) taken at 24 h post-infection at magnification of 20X. The number of transfected cells determined by multiple independent round of counting.

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**Suppression of exogenous target gene expression by miRNA**

To examine the functionality of miR strategy, a preliminary experiment was performed by either co-transfection of pmiR/*lacZ* and *lacZ* reporter plasmid or pmiR and *lacZ* reporter plasmid into cultured cells. The transfected cells were harvested and analyzed for inhibition of β-gal expression by Western blot analysis at 48 h post-transfection. It was found that β-gal expression was completely inhibited in cells co-transfected with pmiR/lacZ and *lacZ* reporter plasmid, as shown in Figure 2A lane 3, while inhibitions were not observed in cells co-transfected with pmiR and *lacZ* reporter plasmid (see Figure 2A lane 4). Our results indicated the efficiency and specificity of the miRNA targeting. In addition, it was also suggested the strong correlation of EmGFP expression with the inhibition of the target gene by miRNA (see Figures 1A and 2A).

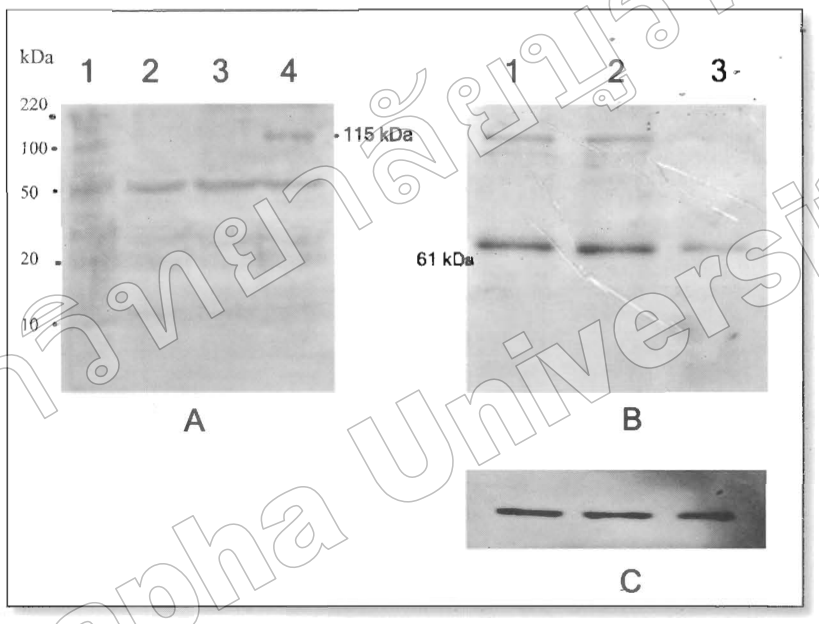


**Figure 2A.** Western blot analysis with anti-β gal IgG of cell lysates harvested 48 h after transfection in HeLa cells with either pmiR (lane 2) or co-transfect with pmiR/*lacZ* and *lacZ* reporter vector (lane 3) or pmiR and *lacZ* reporter vector (lane 4). The expected molecular weight of β-galactosidase protein was shown at 115 kDa compared with the marker (lane 1).

**Figure 2B.** Western blot analysis with either anti-RIP/Rab or anti-β-actin IgG, respectively of cell lysates har vested 48 h after transfection in 293T cells with pmiR (lane 1), or pmiR/*hRIP* 757 (lane 2), or pmiR/*hRIP* 1504 (lane 3). β-actin was used as an internal control (Fig 2C). The expected molecular weight of hRIP protein was shown at 61 kDa.

An effect of miR-mediated interference at the post-transcriptional level was assessed at 48 h post-transfection by Western blot analysis to determine whether the miRNA generated could effectively suppress the expression of endogenous *hRIP* in cultured cells. A moderate reduction in the level of hRIP protein was observed only in cells that were transfected with pmiR/*hRIP*1504 (see Figure 2B lane 3) compared to that with the vector alone (see Figure 2B lane 1). However, the levels of the internal

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control protein, β-actin level, were unchanged (see Figure 2C). The intensity (raw volume = 33,929.27) of the 61-kDa-protein band in lane 3 of Figure 2B that was analyzed by SynGene-gene tool was reduced approximately 65% compared to that in lane 1(raw volume = 83,640.58). The relative levels of the concerned mRNA were also examined in a semi-quantitative RT-PCR analysis. It was found that 293T cells transfected with pmiR/*hRIP*1504 showed a reduction in *hRIP* transcripts (see Figure 3 lane 3) compared to that with pmiR (see Figure 3 lane 1). However, pmiR/*hRIP* 757 did not reduce the abundance of *hRIP* mRNA (see Figure 3 lane 2). Similar phenomena were demonstrated in HeLa cells (data not shown).

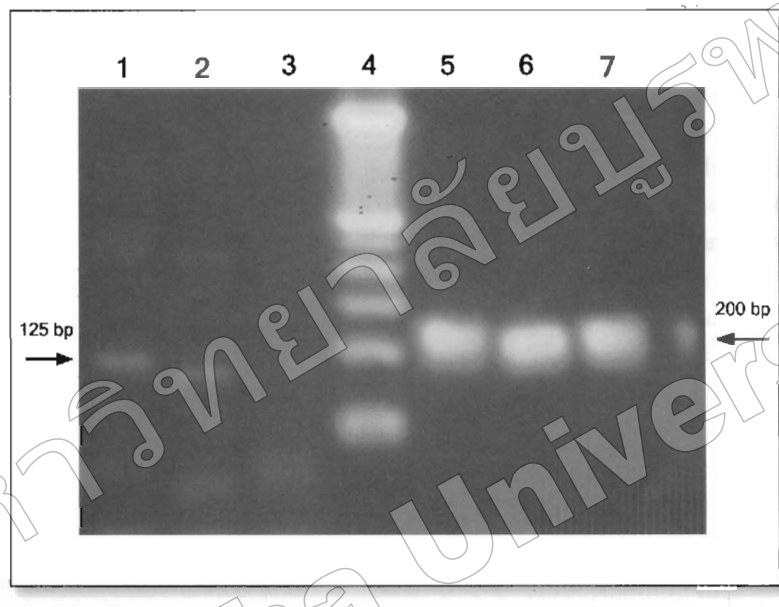


**Figure 3.** An analysis of hRIP mRNA expression in 293 T cells using RT-PCR. RT-PCR for evaluation of RT-PCR analysis of uncleaved *hRIP* mRNA was carried out using *hRIP*-specific primers with concomitant amplification of GADPH. PCR primers were used to amplify a 125 bp fragment in hRIP transcripts shown in Lane 1-3 and a 200 bp fragment in GADPH transcripts shown in Lane 5-7 used as internal control for loading. Lane 1 and 5, relative amount of transcripts in cells transfected with pmiR; Lane 2 and 6, relative amount of transcripts in cells transfected with pmiR/*hRIP*757; Lane 3 and 7, relative amount of transcripts in cells transfected with pmiR/*hRIP*1504 ; Lane 4, 100 bp marker.

## DISCUSSION

The possibility that the expression vectors could be used to express pre-miRNA molecules and specifically inhibit target gene expression in mammalian cells was determined. In this study, miRNA sequences were designed to have a complementary to a distinct region of endogenous *hRIP*mRNA that resulted in target cleavage akin to conventional siRNA. It was shown that a DNA vector based miRNA could specifically inhibit gene expression of the cellular hRIP protein. Both Western blot and RT-PCR experiments consistently suggested that miRNA corresponding to a portion of the *hRIP*mRNA (nt 1504-1524) impaired the expression of endogenous hRIP protein. However, miRNA corresponding to another portion of the *hRIP*mRNA (nt 757-777) did not impair the expression of *hRIP* gene.

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Based on the study on siRNA, the efficacy of each of the siRNA that were designed to target different regions of the mRNA varied greatly (Novena et al., 2002). Thermodynamic characteristics intrinsic to the siRNA strand are a basic determinant of siRNA efficacy (Schubert et al., 2005). In addition, several siRNAs targeting against different sites on the same target mRNA demonstrated striking differences in silencing efficacy (Holen et al., 2002). The low activity of the majority of the siRNA may be due to position effect, or non-accessibility of mRNA for cleavage, caused by higher order RNA structures or protein coverage (Elbashir et al., 2001). The position effects on the endogenous target were more pronounced than on transgene expression (Holen et al., 2002). This observation agreed with our results when β-gal expression was completely inhibited in cells co-transfected with pmiR/*lacZ* and *lacZ* reporter plasmid (see Figure 2A lane 3), whereas moderate suppression of endogenous hRIP expression was demonstrated in cells transfected with pmiR/*hRIP*1504. Thus, features of the target molecule and characteristics inherent to the sequence of the RNAi effector itself can both be limiting factors of silencing efficacy. Sequences of miRNA in this study should be optimized to refine target mRNA-accessible sequences for further improving the diminution of target transcript and protein levels. Moreover, the half-life, the strict control of the interest protein, and an equilibrium kinetic balance between mRNA production and miRNA-mediated mRNA depletion should be taken into account before the use of RNAi technology (Holen et al., 2002).

In conclusion, this study provides evidence that miRNA could be employed as a potential tool to inhibit gene expression of the cellular hRIP protein. However, a side effect of cytotoxicity caused by transfection remains the major hurdle in this study, thereby limiting utility over the use of miRNA in viral-based therapy. If such limitation is overcome, the miRNA could be further investigated in an interference of HIV replication.

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