Short-hairpin RNAs synthesized by T7 phage polymerase do not induce interferon

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ABSTRACT

RNA interference (RNAi) mediated by small-interfering RNAs (siRNAs) is a highly effective gene-silencing mechanism with great potential for gene-therapeutic applications. siRNA agents also exert non-target-related biological effects and toxicities, including immune-system stimulation. Specifically, siRNA synthesized from the T7 RNA polymerase system triggers a potent induction of type-I interferon (IFN) in a variety of cells. Single-stranded RNA also stimulates innate cytokine responses in mammals. We found that pppGn ($n = 2,3$) associated with the 5′-end of the short-hairpin RNA (shRNA) from the T7 RNA polymerase system did not induce detectable amounts of IFN. The residual amount of guanine associated with the 5′-end and hairpin structures of the transcript was proportional to the reduction of the IFN response. Here we describe a T7 pppGn ($n = 2,3$) shRNA synthesis that does not induce the IFN response, and maintains the full efficacy of siRNA.

INTRODUCTION

RNA interference (RNAi) is a natural biological phenomenon mediated by small-interfering RNA (siRNA) molecules that target-specific messenger RNA (mRNA) for degradation by cellular enzymes. RNAi has become the method of choice for studying gene function, especially in mammalian systems. With proof-of-concept studies already reported against a wide variety of human pathogens, and several innovative methods available for delivering siRNA to a range of primary cells, there is now an even greater role for siRNA as a potential therapeutic strategy. siRNAs induce the global upregulation of interferon (IFN)-stimulated gene expression (1–4), as shown by the transfection of both enzymatically and chemically synthesized siRNAs into cells, and by siRNAs that are produced intracellularly following the expression of short-hairpin RNAs (shRNAs) (2–4). These studies documented significant non-specific changes in gene expression as a consequence of the delivery of siRNAs.

One simple method to limit the risk of inducing an IFN response is to use the lowest effective dose of the shRNA vector, as advocated by Bridge et al. (1). Recently, Kim et al. (5) demonstrated that siRNAs synthesized using the T7 RNA polymerase system trigger a potent induction of IFN-α and IFN-β in a variety of cells. The mediator of this response is an initiating 5′-triphosphate that is required for IFN induction. These findings led to the development of an improved method for bacteriophage polymerase-mediated siRNA synthesis that incorporates two 3′-adenosines to prevent base-pairing with the initiating guanines, thereby allowing RNase T1 and calf intestine alkaline phosphatase (CIP) to remove the initiating 5′-nucleotides (nt) and triphosphates of the transcripts. It is now clear that triphosphates act as triggers to induce type I IFN via the activation of retinoic acid-inducible protein I (RIG-I) (6,7). By contrast, Marques et al. (8) reported that blunt siRNAs are potent activators of RIG-I-mediated type I IFN induction, whereas siRNAs containing 3′-overhangs are not.

Here, we describe a new type of shRNA, pppGn($n = 2$)-shRNA, synthesized by bacteriophage polymerase, which does not induce IFN. We also describe the anti-human immunodeficiency virus type 1 (HIV-1) activity of this shRNA, which targets the well-conserved dimerization initiation site (DIS) of HIV-1.

MATERIALS AND METHODS

RNAs

T7 shRNAs were synthesized using the AmpliScribe T7 High Yield Transcription Kit (Epicentre, Madison,

WI) according to the manufacturer's protocol. For the *in vitro* transcription of RNA, T7 primer I (5′-TAATACGACTCACTATA-3′) was hybridized with T7 primer II, which contains the antisense sequence of each transcribed RNA, the tail sequence (5′-CCTATA GTGAGTCGTATTA-3′) and the loop sequence (5′-TC TCTTGAA-3′). For example, to make pppGG-luc3-shRNA, two primers were used (5′-TAATACGACTC ACTATA-3′) and (5′-AAGGAGCCTTCAGGATTACA AGATCTCTTGAATCTTGTAATCCTGAAGGCTCC CCTATAGTGAGTCGTATTA-3′). After a 1-h at 37°C, for the CIP, 50 U RNA (New England Biomedical, Beverly, MA) was added to the shRNA and incubated at 37°C for 1 h. Finally, the shRNAs and siRNA were purified by polyacrylamide gel electrophoresis (PAGE).

Cell culture

HeLa CD4$^+$ cells were grown in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Assays for IFN

The amount of IFN-β secreted into the growth medium was determined using IFN ELISA kits (PBL Biomedical Laboratories, Piscataway, NJ). HeLa CD4$^+$ or RIG-I silencing HeLa CD4$^+$ cells were transfected with 100 nM shRNAs or siRNA using DMRIE-C (Invitrogen, San Diego, CA) according to the manufacturer's protocol, and were placed in 12-well plates. After 90 min, the cells were washed with RPMI-1640. Medium from RNA-transfected HeLa CD4$^+$ cells was collected 12 h later, serially diluted and assayed for the amount of secreted IFN according to the manufacturer's protocol. Each assay was performed in triplicate. Antibody neutralization assays were carried out by diluting the medium to 3.3% with fresh medium and mixing with 100 U/ml IFN-β neutralizing antibody (PBL Biomedical Laboratories) for 1 h.

Dual-luciferase reporter assay

HeLa CD4$^+$ cells in 12-well plates were co-transfected with 0.2 μg firefly luciferase vector, pGL3 control vector or pNL$_{4-3}$-luc, and 0.2 μg pRG-TK control vector containing Renilla luciferase (Promega Corp. Madison, WI) using FuGENE^{TM}6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. shRNAs were used at a concentration of 12.5–50 nM per well. Firefly and Renilla luciferase activities were measured consecutively using dual-luciferase assays (Promega) 48 h after transfection.

Assay of HIV-1 replication

HIV-1 production was monitored by determining the HIV-1 p24 antigen concentration. HeLa CD4$^+$ cells were transfected with shRNAs or LacZ (control shRNA) using DEMRI-C (Invitrogen). After 90 min, the cells were washed with serum-free RPMI-1640 and then transfected with pNL$_{4-3}$ using FuGENE^{TM}6 (Roche Diagnostics)

according to the manufacturer's protocol. The culture medium from HeLa CD4$^+$ cells was harvested 2 days post-transfection. p24 Gag protein production was detected by the HIV-1 p24 chemiluminescent enzyme immunoassay (CLEIA; Lumipulse, Fujirebio Inc., Tokyo, Japan), according to the manufacturer's protocol (9).

Dual-luciferase reporter assay of IRF-3 activity

HeLa CD4$^+$ cells were seeded at a density of 1×10^5 cells per well into 12-well plates for 24 h, and then transfected with 0.5 μg pIRF-3/Luc DNA (10) and 0.2 μg phRG-TK using FuGENE^{TM}6 (Roche Diagnostics) according to the manufacturer's protocol. After 24 h, 100 nM shRNA or 0.5 μg/ml polyinosinic acid:polycytidylic acid (poly I:C) was transfected using DMRIE-C (Invitrogen). Untreated cells were used as a control. Firefly and Renilla luciferase activities were measured consecutively using dual-luciferase assays (Promega Corp.) 12 h after transfection.

MTS assay

HeLa CD4$^+$ cells (2×10^4 cells/ml) were seeded into 96-well microtitre plates and incubated in the presence of various concentrations of the test compounds. The dilutions ranged from one-fold to five-fold, and nine concentrations were examined. All of the experiments were performed in triplicate. After 3 days culture at 37°C in a CO_2 incubator, cell viability was quantified by a colorimetric assay monitoring the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Promega Corp.) to a red formazan product (11). The absorbances were read by a microcomputer-controlled photometer (Titertec MultiscanR; Labsystem Oy, Helsinki, Finland) at a single wavelength (492 nm). These values were then translated into percentages per well.

RNA interference in HeLa CD4$^+$ cells

HeLa CD4$^+$ cells were transfected with 100 nM siRNAs targeting RIG-I (sense CGAUUCCAUCACUAUCC AUtt; antisense, AUGGAUAGUGAUGGAAUCGtt; Sigma Aldrich Japan, Hokkaido, Japan) using DMRIE-C (Invitrogen) according to the manufacturer's protocol, and were placed in 12-well plates.

Reverse-transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was prepared using TRIZOL (Invitrogen). RIG-ImRNA was detected by a RT-PCR High–Plus kit (Toyobo, Kyoto, Japan) with the following specific primers: RIG-I sense, 5′-TCCTTTATGAGTAT GTGGGCA-3′; and RIG-I antisense, 5′-TCGGGCACA GAATATCTTTG-3′. Glyceraldehyde-3-phosphate dehy-drogenase (GAPDH)-specific primers were used as a loading control in a separate reaction. The reaction parameters were 2 min at 94°C, followed by 40 cycles of 1 min at 94°C and 1.5 min at 60°C.

RESULTS

pppGn($n = 2$)-shRNA does not induce IFN

To investigate the RNAi-mediated silencing of luciferase activity, we initially synthesized six shRNAs targeting the luciferase gene transcript using T7 RNA polymerase. The main advantages of this technique are its simplicity and low cost compared with chemical synthetic RNA (12,13). The luc-shRNAs include a 5′-pppGn ($n = 2$) sequence, because efficient T7 RNA polymerase initiation requires the first and second non-transcribed spacer of each RNA to be a guanine.

To determine whether pppGn($n = 2$)-shRNA-luc specifically inhibited luciferase gene expression, HeLaCD4$^+$ cells were transfected with pppGn($n = 2$)-shRNA-luc corresponding to the luciferase gene, and then transfected with pGL3-control (Firefly) and phRG-TK (Renilla) vectors. We found that pppGn($n = 2$)-shRNA-luc3 (nt 824–845) and pppGn ($n = 2$)-shRNA-4 (nt 893–914) greatly inhibited luciferase activity (data not shown).

Recently, Kim *et al.* (5) demonstrated that siRNAs synthesized using the T7 RNA polymerase system trigger the potent induction of IFN-α and IFN-β in a variety of cells. The mediator of this response was an initiating 5′-triphosphate that was required for IFN induction. To verify the induction of IFN by pppGn($n = 2$)-shRNA-luc3, we designed pppGn ($n = 0$–3) in association with the 5′-end of shRNA-luc3, which was transcribed by T7 RNA polymerase (Figure 1A). Luc-3 inhibited luciferase activity in a dose-dependent manner (Figure 1B). The control pppGn($n = 2$)-shRNA-EGFP targeting enhanced green fluorescent protein did not inhibit luciferase activity (Figure 1B). Furthermore, the inhibition of luciferase activity was not due to the residual amount of guanine associated with the 5′-end of the transcript.

We also designed 5′-HOGn($n = 0$–3)-luc-3 with the 5′-end of shRNA and tested for IFN induction (Figure 1A). We assayed the medium of pppGn or 5′-HOGn($n = 0$–3)-shRNA-luc3-transfected HeLa CD4$^+$ cells for IFN-β using an enzyme-linked immunosorbent assay (ELISA). IFN assays from pppGn ($n = 2,3$) associated with the 5′-end of shRNA did not induce IFN in HeLa CD4$^+$ cells (Figure 2A), whereas a slight IFN response was induced by pppGn ($n = 1$) associated with the 5′-end of shRNA. pppGn ($n = 0$) associated with the shRNA 5′-end was capable of more potent IFN induction than pppGn ($n = 1$) associated with the shRNA 5′-end. Furthermore, no IFN was induced in HeLa CD4$^+$ cells by 5′-HOGn ($n = 0$–3) (Figure 2A).

To further investigate the pppGn($n = 0$–3)-shRNA-luc3-mediated or 5′-HOGn($n = 0$–3)-shRNA-luc3-mediated IFN response, we monitored cell growth. pppGn($n = 0$)-shRNA-luc3-transfected cells showed a cytopathogenic effect after 3 d, but pppGn($n = 2,3$)-shRNA-luc3-transfected cells did not (Figure 2B). A tetrazolium-based MTS assay was next used to examine the viability of pppGn($n = 0$–3)-shRNA-luc3-transfected or 5′-HOGn(0–3)-shRNA-luc3-transfected cells. pppGn ($n = 2,3$) associated with the shRNA 5′-end or 5′-HOGn(0–3)-shRNA-luc3 did not induce cellular toxicity
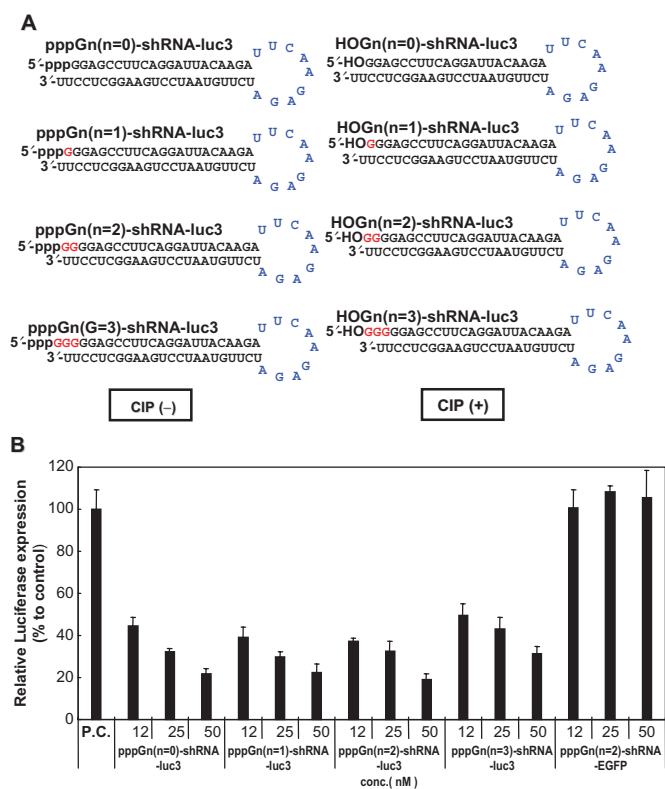

Figure 1. RNAi-mediated silencing of luciferase activity. (**A**) The shRNA-luc synthesized in these studies. We designed pppGn ($n = 0$–3) associated with the 5′-end of the shRNA that was transcribed by T7 RNA polymerase. We also designed 5′-HOGn ($n = 0$–3) with the 5′-end of the shRNA (removal of triphosphate by CIP). (**B**) The anti-luciferase activity of pppGn ($n = 0$–3) associated with the 5′-end of shRNA-luc3 in HeLaCD4$^+$ cells. Firefly and Renilla luciferase activities were measured consecutively using dual-luciferase assays (Promega) 48 h after transfection. The average results of three independent experiments are presented.

in HeLa CD4$^+$ cells (Figure 2C), whereas minor cellular toxicity was induced by pppGn($n = 0,1$)-shRNA-luc3.

The antiviral activities of IFN have been recently studied (14), but our data indicate that the presence of a guanine residue on T7-transcribed RNAs prevents the activation of IFN. Furthermore, the inhibition of luciferase activity by pppGn($n = 0$)-shRNA-luc3 (Figure 2A–C) might be due, in part, to IFN induction. These results suggest that the residual amount of guanine associated with the 5′-end of the transcript is proportional to the reduction of the IFN response.

Inhibition of HIV-1 replication by shRNAs synthesized using T7 RNA polymerase

To confirm the role of residual guanine, we tested the sequence-specific inhibition of HIV-1 replication by shRNAs synthesized using the T7 RNA polymerase. We applied the shRNA technology to a well-conserved target in HIV-1: the dimerization initiation site (DIS) (15,16). The DIS is a stem–loop structure with six self-complementary nt at the top (Figure 3A), which is located between the primer binding site and the splice donor site at the end of a long terminal repeat (LTR) (17). It is involved
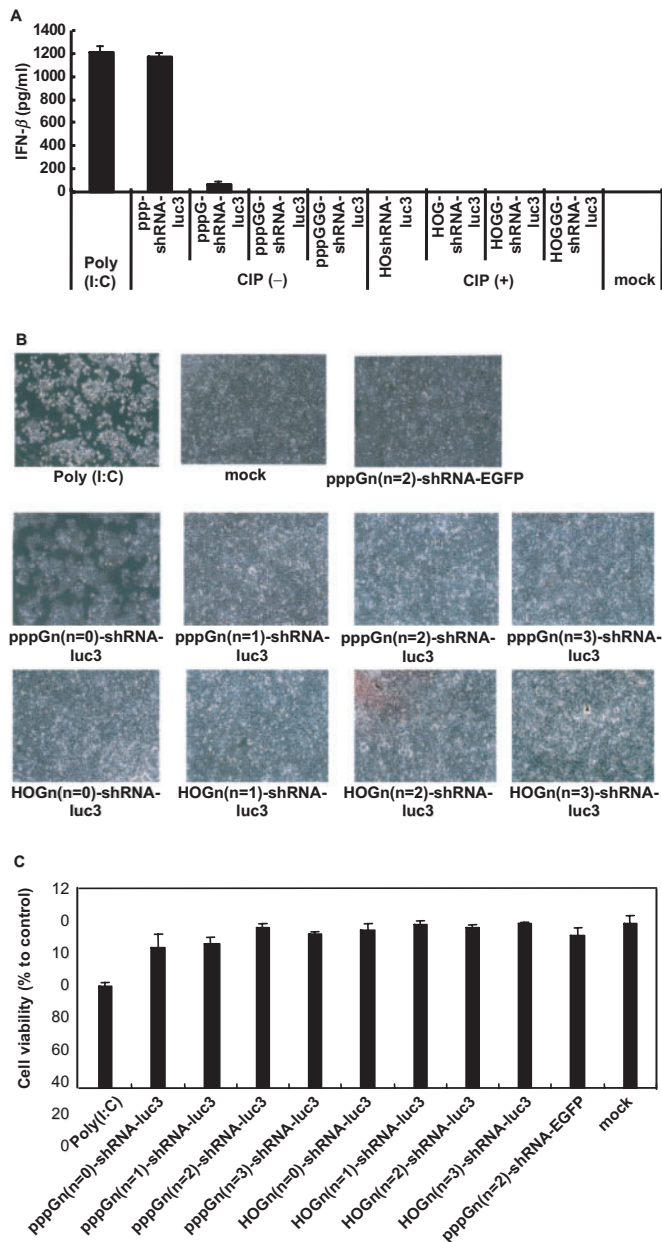
Figure 2. Lack of IFN induction by T7-transcribed shRNA. (**A**) The residual amount of guanine associated with the 5′-end of the transcript is essential to prevent IFN induction. HeLaCD4$^+$ cells were transfected with 100 nM of pppGn(n = 0–3)-shRNA-luc3 or HOGn(n = 0–3)-shRNA-luc3. The induced levels of IFN-β were determined by an ELISA. The IFN results are the average of two independent experiments. (**B**) The cytopathic effect of T7-transcribed pppGn(n = 0–3)-shRNA-luc3. HeLaCD4$^+$ cells were transfected with 100 nM of pppGn(n = 0–3)-shRNA-luc3 or HOGn(n = 0–3)-shRNA-luc3 and monitored microscopically 3 days post-transfection. (**C**) Cytotoxicity of pppGn(n = 0–3)-shRNA-luc3 (100 nM) or HOGn(n = 0–3)-shRNA-luc3 (100 nM) represents the percentage reduction of viable cell numbers in HeLaCD4$^+$ cells. The cytotoxicity assay was performed with an MTS assay. The toxicity results are representative of three independent experiments.

in the dimerization of the HIV-1 genome, packaging and proviral synthesis (18–22). There are two major motifs in HIV-1: GUGCAC in subtypes A and C, and GCGCGC in subtypes B and D (23,24). The subtype A motif has been

shown to be a good siRNA target in an *in vitro* cell-free system where HIV-1 genome dimerization was successfully inhibited by a 9-mer DIS-targeting siRNA, while the corresponding DNA oligonucleotide did not affect dimerization (25,26). These studies also showed that sense oligonucleotides function as competitive inhibitors of this self-complimentary target.

pppGn(n = 2)-shRNA-DIS (shRNA-DIS-1-8) targeted against the 35-nt stem-loop (nt 248–277) or the 9-nt loop of the DIS (nt 255–263) were tested for their ability to inhibit HIV-1 replication (Figure 3B). The effect of shRNA-DIS-1-8 on HIV-1 (pNL$_{4-3}$-luc) (27) replication was measured in a transient assay following its co-transfection with pNL$_{4-3}$-luc into HeLa CD4$^+$ cells. At 48 h post-transfection, luciferase activity was measured with the dual-luciferase reporter (DLR^{TM}) assay system. The luciferase activity of the cell lysate was measured as an indirect marker of viral replication. Although shRNA-DIS-3, shRNA-DIS-7 and shRNA-DIS-8 had strong inhibitory effects compared with shRNA-DIS-1 and shRNA-DIS-2, their anti-HIV-1 activities were lower than those of shRNA-DIS-4, shRNA-DIS-5 and shRNA-DIS-6 (Figure 3C). The internal control, pppGn(n = 2)-shRNA-LacZ targeting LacZ protein, did not inhibit luciferase activity.

Sequence-specific inhibition of HIV-1 replication by T7 transcribed shRNA-DIS-6

To further investigate the specific effect of shRNA-DIS6 on the DIS sequence, we examined shRNA-DIS-6 and the internal control shRNA-LacZ against infection by HIV-1$_{NL4-3}$ (Figure 4A). The shRNA-DIS effect was confirmed as specific to the DIS gene, because no obvious effects were observed following the HIV-1$_{NL4-3}$ challenge.

We next carried out an IFN-β ELISA assay of the medium of pppGn(n = 2)-shRNA-DIS6-transfected, HOGn(n = 0)-shRNA-DIS6-transfected, and ppp Gn(n = 2)-shRNA-LacZ-transfected HeLa CD4$^+$ cells, and found no IFN induction (Figure 4B). By contrast, pppGn (n = 0) associated with the shRNA 5′-end induced IFN. Kim *et al.* (5) reported that the pppGGG-dsRNA (such as small interfering RNA), when generated by *in vitro* transcription, induced IFN. We examined the influence of pppGGG associated with short dsRNA [pppGn(n = 2)-siRNA-DIS6] on IFN inducing activity. When HeLa CD4$^+$ cells were transfected with pppGn(n = 2)-siRNA-DIS6, we observed IFN induction, but not the pppGn(n = 2)-shRNA-DIS6 (Figure 4B). Our result on the induce of IFN by pppGn(n = 2)-siRNA-DIS6 was in general agreement with their IFN response as reported by Kim *et al.* (5). To further clarify the IFN response of pppGn (n = 0,2) in association with the shRNA 5′-end, we examined the activation of IFN-regulatory factor-3 (IRF-3) in HeLa CD4$^+$ cells. We assessed the trigger of IRF-3 phosphorylation by pppGn(n = 0,2)-shRNA-DIS6 and HOGn(n = 0)-shRNA-DIS6 in HeLa CD4$^+$ cells by constructing a luciferase reporter gene-expression vector (pIRF-3/Luc reporter) with an IRF-3 binding region (5′-GAAACCGA AACT-3′) in the pGL3-basic vector (28). pIRF-3/Luc and
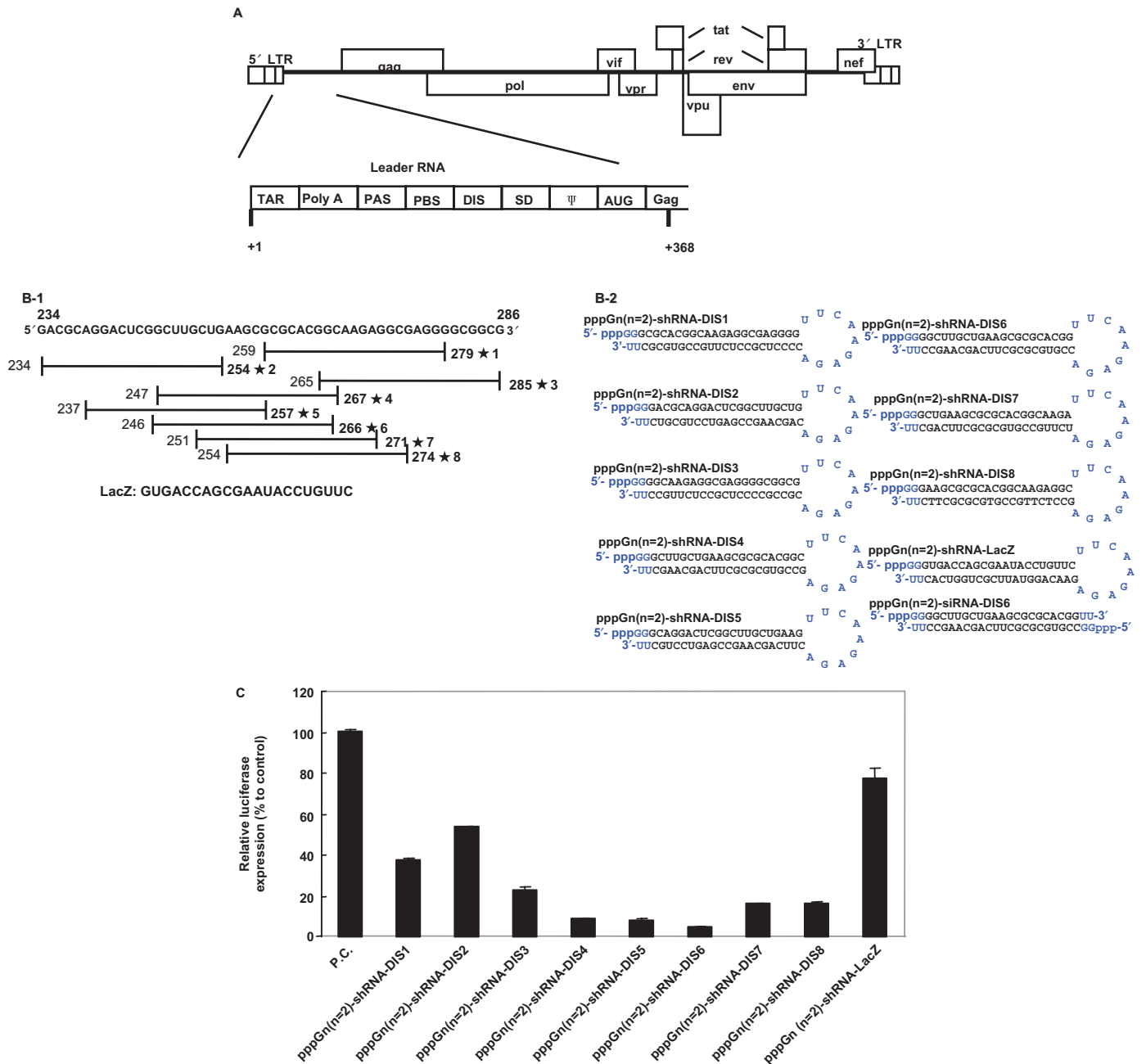
Figure 3. Inhibition of HIV-1 replication by shRNAs synthesized using T7 RNA polymerase. (**A**) Schematic of the 9.2-kb HIV genome. The 5′-LTRs and 3′-LTRs and all eight open-reading frames are indicated. The untranslated leader RNA consists of several regulatory domains. (**B**) shRNAs or siRNA targeted against the DIS of HIV-I RNA (nt 248–277). (**C**) The effect of shRNA-DIS1-8 on HIV-1 (pNL$_{4-3}$-luc) replication was measured in a transient assay following its co-transfection with pNL$_{4-3}$-luc into HeLa CD4$^+$ cells. Firefly and Renilla luciferase activities were measured consecutively using dual-luciferase assays (Promega) 48 h after transfection. The luciferase activity of the cell lysate was measured as an indirect marker of viral replication. The average results of three independent experiments are presented.

pppGn($n = 0,2$)-shRNA-DIS-6 were then co-transfected into HeLa CD4$^+$ cells. IRF-3 activation was monitored using the DLR^{TM} assay (Figure 4C). The internal control, polyI:C and pppGn($n = 0$)-shRNA-DIS6 simultaneously induced phosphorylation of IRF-3 and luciferase gene expression in HeLa CD4$^+$ cells. By contrast, pppGn($n = 2$)-shRNA-DIS6 and HOGn($n = 0$)-shRNA-DIS6 did not mediate either IRF-3 phosphorylation or luciferase gene expression in these cells (Figure 4C).

To further clarify the RIG-I-mediated IFN response to pppGn($n = 0,2$)-shRNA, we used RNAi to specifically target RIG-I in HeLa CD4$^+$ cells. After 2 days, cells were transfected with shRNAs [pppGn ($n = 0,2$) or HOGn($n = 0$)-shRNA-DIS6] using DMRIE-C, and the IFN ELISA assay were carried out. However, pppGn ($n = 0,2$)-shRNA-DIS6 and HOGn($n = 0$)-shRNA-DIS6 strongly inhibited INF-β production (Figure 4D). These data indicate that pppGn($n = 0$)-shRNA induced an
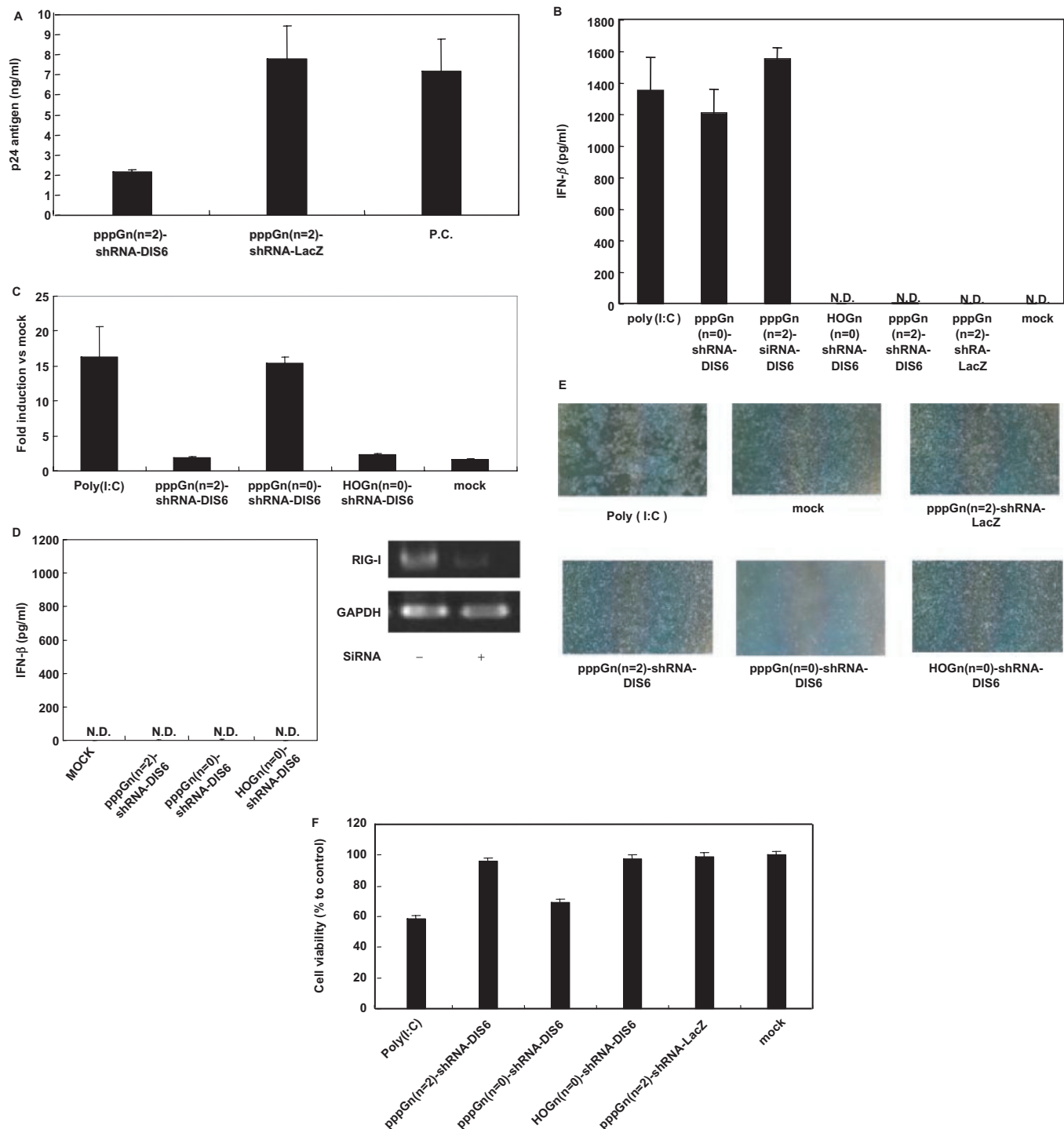
Figure 4. Sequence-specific inhibition of HIV-1 replication by T7-transcribed shRNA-DIS-6. (**A**) T7-transcribed pppGn($n = 2$)-shRNA-DIS6 and LacZ (control) against challenge infection of HIV-1$_{NL4-3}$. The average results of three independent experiments are presented. (**B**) The residual amount of guanine associated with the 5′-end of the transcript is essential to prevent the induction of IFN. HeLaCD4$^+$ cells were transfected with 100 nM of either pppGn($n = 0,2$)-shRNA-DIS6, HOGn($n = 0$)-shRNA-DIS6 and pppGn($n = 2$)-shRNA-LacZ (control) or pppGn($n = 2$)-siRNA-DIS6 (control). The induced levels of IFN-β was determined by an ELISA. ND, not detectable. The IFN results are the average of two independent experiments. (**C**) Activation of IRF-3 by T7-transcribed shRNA. HeLaCD4$^+$ cells transfected with pIRF-3/Luc plasmid and Renilla luciferase control plasmids were either pppGn($n = 0,2$)-shRNA-DIS6 and HOGn($n = 0$)-shRNA-DIS6. After 12 h, the cell lysates were prepared and assayed for dual luciferase activity. The average results of three independent experiments are presented. (**D**) RIG-I siRNA inhibited the IFN-β response by pppGn($n = 0,2$)-shRNA-DIS6 or HOGn($n = 0$)-shRNA-DIS6. HeLaCD4$^+$ cells were transfected with siRNA targeting RIG-I. After 2 d, HeLaCD4$^+$ cells were transfected with 100 nM of either pppGn($n = 0,2$)-shRNA-DIS6 or HOGn($n = 0$)-shRNA-DIS6. The induced levels of IFN-β were determined by an ELISA. N.D., not detectable. The IFN results are the average of three independent experiments. (**E**) Cytotoxic effect of T7-transcribed pppGn($n = 2$)-shRNA-DIS6 or pppGn($n = 2$)-shRNA-LacZ. HeLaCD4$^+$ cells were transfected with pppGn($n = 2$)-shRNA-DIS6 or pppGn($n = 2$)-shRNA-LacZ (control) and monitored microscopically 3 days post-transfection. (**F**) The cytotoxicity of pppGn($n = 0,2$)-shRNA-DIS-6 or HOGn($n = 2$)-shRNA-DIS6 represented as the percentage reduction of viable HeLaCD4$^+$ cells. A cytotoxicity assay was performed along with an MTS assay. The toxicity results are representative of the three independent experiments.

RIG-I-mediated INF response, whereas $pppGn(n = 2)$-shRNA arrested INF-inducing activity through TLR-3 and RIG-I.

We next monitored cell growth. The $pppGn(n = 0)$-shRNA-DIS6-transfected cells showed cytopathogenic effects after 3 days, but the $pppGn(n = 2)$-shRNA-DIS6-transfected cells did not (Figure 4E). Finally, we used the MTS assay to examine the viability of $pppGn(n = 0,2)$-shRNA-DIS-6-transfected HeLa $CD4^+$ cells. $HOGn$ $(n = 2)$-shRNA-DIS-6 and $pppGn(n = 2)$-shRNA-DIS-6 or LacZ did not induce cellular toxicity (Figure 4E), whereas control polyI:C and $pppGn(n = 0)$-shRNA-DIS-6 did (Figure 4F). Together, these data indicate that the residual guanine associated with the 5′-end and the hairpin-loop structures of the transcript is proportional to the reduction of the IFN response.

DISCUSSION

The present study demonstrates that the previously proposed scheme of triphosphate-dependent siRNA recognition (5) is, in fact, reversed; although T7-transcribed-siRNA induced a vigorous type I IFN response, 5′OH-siRNA did not.

Our studies raise the question of how the residual guanine that is associated with the 5′-end of the transcript contributes to reducing the IFN response. To investigate the RNAi-mediated silencing of luciferase activity, we synthesized shRNA-luc, including the 5′-$pppGn$ $(n = 0–3)$ sequence, because efficient T7 RNA polymerase initiation requires the first and second non-transcribed space of each RNA to be a guanine. The $pppGn$ $(n = 2,3)$ associated with the 5′-end of shRNA does not induce IFN in HeLa $CD4^+$ cells (Figure 2A), whereas the IFN response was weakly initiated by the $pppGn$ $(n = 1)$ associated with the 5′-end of the shRNA. The $pppGn$ $(n = 0)$ associated with the 5′-end of the shRNA more potently induced IFN than the $pppGn$ $(n = 1)$ associated with the 5′-end of the shRNA. Furthermore, 5′-$HOGn$ $(n = 0–3)$ did not induce IFN in HeLa $CD4^+$ cells. Our results suggest that the residual amount of guanine associated with the 5′-end of the transcript is proportional to the reduction of the IFN response. In another study, T7-transcribed siRNAs triggered a potent induction of IFN-α and IFN-β in a variety of cells, and the mediator of this response was an initiating 5′-triphosphate that was required for IFN induction (5). More recently, two groups reported that type I IFN is induced by the activation of RIG-I by 5′-triphosphate RNA (6,7). It will be important to re-examine these data in light of the possibility that these RNAs contain triphosphates, which complicates the interpretation. Our data indicate that 5′-triphosphates are responsible for inducing IFN activity, whereas the residual amount of guanine associated with the 5′-end of the transcript arrests the IFN response. To reconfirm the role of the residual amount of guanine associated with the 5′-end of the shRNA, we tested the sequence-specific inhibition of HIV-1 replication by shRNAs synthesized using T7 RNA polymerase. The HIV genome is a homo-dimer of two sense RNA single strands. We applied the shRNA technology to a well-conserved target in HIV-1, the DIS (15,16). The $pppGn(n = 2)$-shRNA-DIS6, as the target of the DIS gene, was a more potent inhibitor than the other types of shRNA-DIS. The synthesized $pppGn(n = 2)$-shRNA-DIS6 did not induce the activation of IFN (Figure 4B), but not the $pppGn(n = 0)$-shRNA-DIS6 and $pppGn(n = 2)$-siRNA-DIS6. Furthermore, the activation of IRF-3 did not induce with the $pppGn(n = 2)$-shRNA-DIS6 and $HOGn(n = 0)$-shRNA-DIS6, but not the $pppGn(n = 0)$-shRNA-DIS6 (Figure 4C). However, RIG-I-siRNA transfected cells which did not stimulation of IFN by both $pppGn$ $(n = 0)$ or $pppGn(n = 2)$-shRNA-DIS6 (Figure 4D). Recently, it was demonstrated that the pppGGG associated with short double-stranded RNA (dsRNA; pppGGG-dsRNA) is recognized by RIG-I but not TLR-3 (5,6). In contrast, the $pppGn(n = 2)$-shRNA-DIS6 also included pppGGG on the 5′-end of the shRNA, but did not recognize TLR-3 and RIG-I (Figure 4B–D). Furthermore, $pppGn(n = 2)$-shRNA-DIS6 presented a hairpin-loop structure together with the 5′-pppGGG, but not the presence on pppGGG-dsRNA. Hence, the pppGGG dsRNA was capable of inducing IFN (5,6), but the $pppGn(n = 2)$-shRNA-DIS6 with the guanine associated with the 5′-end and the hairpin-loop structures precluded the IFN response. One study suggested that synthetic short dsRNA with blunt ends is recognized by RIG-I, and that 2-nt overhangs at the 3′-end block this recognition (7). Taken together, these data indicate that the mechanism of inducing IFN by dsRNA with 5′-pppGGG can differentiate between dsRNAs with or without the guanine associated with the 5′-end and hairpin-loop structures. These results demonstrated an association with sequence-specific inhibition via the RNAi mechanism.

In conclusion, shRNAs transcribed by T7 RNA polymerase activate cells of the immune system cells and induce IFN production. However, the presence of a triphosphate on *in vitro*-transcribed RNAs can induce activation of the immune system (5). The $pppGn$ $(n = 2–3)$ associated with the 5′-end of the shRNA from T7 RNA polymerase did not induce detectable IFN, whereas $pppGn$ $(n = 0,1)$ associated with the 5′-end of the shRNA did induce IFN. Importantly, the residual amount of guanine associated with the 5′-end and the hairpin-loop structures of the transcript was proportional to the reduction of the IFN response. The improved method of T7 RNA polymerase-mediated shRNA synthesis did not require RNase treatment to remove the initiating 5′-nt and triphosphates of the transcripts. Our identification of a putative immunostimulatory motif with shRNAs provides a basis for the rational design of synthetic shRNAs that avoid inducing an immune response. This finding will help to reduce the potential for off-target gene effects.

Conflict of interest statement. None declared.

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