RESEARCH ARTICLE

Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region

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Journal of RNAi and Gene Silencing (2005), 1(2), 66-78

(Received 01 July 2005; Revised 20 September 2005; Accepted 26 September 2005, Available online 11 October 2005; Published 14 October 2005)

ABSTRACT

In addition to the degradation of homologous RNAs through the RNA interference (RNAi) pathway, small interfering RNAs (siRNAs) can in some systems induce cytosine methylation and transcriptional silencing of homologous promoters. Targeting of HIV-1 by RNAi results in transient suppression of the virus through degradation of viral transcripts. In an effort to prolong the suppressive effect of siRNAs on productive HIV-1 infection, we targeted conserved tandem NF-κB binding motifs in the viral LTR. A 21-nucleotide-RNA duplex induced marked and durable (at least 30 days) suppression of productive HIV-1 infection in chronically infected MAGIC-5 cells. This suppression is associated with CpG methylation within the 5'LTR and marked reduction of HIV-1 transcription in nuclear run-on assays. We then assessed three additional siRNAs targeting other sites within the HIV-1 promoter region. These siRNAs suppressed HIV-1 infection to different extents and the degree of suppression correlated with the extent of *de novo* methylation of CpG motifs within the HIV-1 promoter region. These findings indicate that HIV-1 can be silenced by an RNA-directed mechanism that suppresses transcription and induces CpG methylation. In addition to providing evidence that this RNA-directed DNA methylation is active in mammalian cells, this is the first report of prolonged suppression of HIV-1 infection induced by siRNA.

KEYWORDS: *siRNA, RNAi, gene-silencing, HIV-1, DNA-methylation, transcriptional silencing, TGS*

INTRODUCTION

RNA interference (RNAi) can suppress a range of pathogenic human viruses including poliovirus (Gitlin et al, 2002), hepatitis virus (Chang and Taylor, 2003; Giladi et al, 2003; Randall et al, 2003; Yokota et al, 2003), human papillomavirus (Jiang and Milner, 2002) and HIV-1 (Capodici et al, 2002; Coburn and Cullen, 2002; Jacque et al, 2002; Lee et al, 2002; Novina et al, 2002; Stevenson, 2003). Small interfering RNAs (siRNAs) of ~22 nucleotides targeting HIV-1 structural and accessory genes induce rapid degradation of mRNA containing complementary sequence, and suppress the production of new virus *in vitro*. The duration of this effect varies from 4 to 7 days (Capodici et al, 2002), which may limit the clinical utility of RNAi-based therapies. However, prolongation of this effect has been achieved using adeno-associated virus or lentiviral vectors to deliver stably expressed siRNA. Using this approach HIV-1 can be suppressed for between 14 and 25 days. However, HIV-1 is known to adapt to environmental pressures and rapid selection of siRNA escape mutants has been described *in vitro* (Boden et al, 2003; Das et al, 2004), potentially further limiting the efficacy of treatment modalities based on this approach.

We sought other means to prolong the suppressive effect of siRNA that would be less susceptible to the adaptability of HIV-1. In plants and certain other model systems in

mammalian cells (Kawasaki and Taira, 2004; Morris et al, 2004), siRNA targeted to promoter regions can induce silencing through a mechanism that seems to be distinct from RNAi. This mechanism involves transcriptional gene silencing (TGS) and cytosine methylation (Mette et al, 2000; Jones et al, 2001) and has been termed RNA-directed DNA methylation (RdDM) (Aufsatz et al, 2002; Pal-Bhadra et al, 2002). While the exact molecular mechanisms of RdDM are unknown, it is likely to involve cytosine methylation, histone modification and chromatin remodeling. Recently, the RNA-induced initiation of transcriptional gene silencing (RITS) complex has been implicated in the formation of heterochromatin. RITS contains both dicer derived siRNA that guides the complex to an homologous nucleic acid sequence, as well as proteins Ago1, Chp and Tas 3 that play a role in the formation of heterochromatin. Together these components have been shown to induce site-specific silencing of centromeric loci in fission yeast. The process is associated with methylation of histone H3 at Lys 9 (Verdel et al, 2004). However, the exact mechanisms by which RITS complex induces and maintains site-specific RdDM are yet to be fully delineated (Noma et al, 2004; Matzke and Birchler, 2005).

Regardless of the exact mechanisms, once induced, RdDM is long lasting and can be passed on across generations in plant systems (Sijen et al, 2001; Burgers et al, 2002; Robertson, 2002) Clearly, the harnessing of such prolonged gene silencing would have advantages for therapeutics based on siRNAs. Furthermore, this approach has another theoretical advantage when the target is highly adaptable. The adaptability of HIV-1 is related to its high turnover rate combined with the rate of base pair mismatches that occurs during reverse transcription. With transcription halted, opportunities for generation of mutations and, therefore, selection of escape variants should be significantly reduced. Recent reports indicate RdDM and short-lived transcriptional gene silencing can be induced in mammalian cells by siRNAs (Kawasaki and Taira, 2004; Morris et al, 2004). However, in these systems silencing required the use of multiple siRNAs simultaneously.

We tested the ability of several siRNAs targeting different sequences within the promoter region of HIV-1 to induce gene silencing and compared their activity to that of a siRNA, which is known act by degradation of homologous RNAs. DNA methylation status in the promoter region and transcriptional activity was assessed using MAGIC-5 cell (CCR5-CXCR4 expressing HeLa/CD4+ cell line) infected with HIV-1.

MATERIALS AND METHODS

RNA duplexes

Double-stranded RNA duplexes were obtained from Dharmacon Research Inc (Lafayette, CO). HIV-1 was targeted with the duplexes:

HIV-prom-A siRNA:
5'-GGGACUUUCCGCUGGGGACTT-3' (sense)
5'-GUCCCCAGCGGAAAGUCCCTT-3' (antisense)
HIV-prom-B siRNA:
5'-GGCCCGAGAGCUGCAUCCGGTT-3' (sense)
5'-CCGGAUGCAGCUCUCGGGCCTT-3' (antisense)
HIV-prom-C siRNA:
5'-GACUGCUGACAUCGAGCUUTT -3' (sense)
5'-AAGCTCGATGTCAGCAGTCTT-3' (antisense)
HIV-prom-D siRNA:
5'-CUGGGGAGUGGCGAGCCCUTT -3' (sense)
5'-AGGGCUCGCCACUCCCCAGTT -3' (antisense)
HIV- gag siRNA:
5'-GAUUGUACUGAGAGACAGGCUTT-3' (sense)
5'-AGCCTGTCTCTCAGTACAATCTT-3' (antisense)
Scrambled siRNA (synthesized with the same overall nucleotide content as HIV-prom-A siRNA):
5'-CAGCUGGGACGUGUGCCUGTT-3' (sense)
5'-CAGGCACACGTCCCAGCTGTT-3' (antisense)

HIV infection and transfection of siRNA

MAGIC-5 cells (2×10^5) were infected with the HIV-1 subtype B strain NL4-3 (50 pg reverse transcriptase, equivalent to 1.3×10^6 copies of HIV-RNA) and infection was allowed to establish for 5 days. On day 6 cells were detached with 0.25% (v/v) trypsin/1mM EDTA, washed twice and re-seeded at 2×10^5 in a 25 ml flask. After 16 hr, the infected cells were transfected using a concentration of 530 pM of one of the synthesized siRNAs using Oligofectamine (Invitrogen, Mount Waverley, Australia) according to the manufacturer's instructions. Cultures were maintained according to standard protocols (Aldovini and Walker, 1990). Briefly, $25cm^2$ flasks were seeded with 2×10^5 cells every 4-6 days. Cultures with high level HIV-replication were supplemented with fresh uninfected cells at each passage. In one set of experiments a range of viruses were used including, HIV-1 laboratory strains HIV-1 IIIB, RF and HIV-2 CBL-20, as well as two primary isolates derived from patients, failing combination anti-retroviral therapy (Kaufmann et al, 2001).

Viral quantification

Reverse transcriptase activity in culture supernatants was determined as previously described (Suzuki et al, 1993). HIV-1 mRNA was quantified using a real-time RT-PCR assay specific for HIV-*gag*. RT-PCR reactions were performed with SuperScript One-step RT-PCR (Invitrogen) using 0.4 μM of both sense and anti-sense primers, and 0.1 μM of sequence-specific fluorogenic Taqman probe. Standard curves were constructed using genomic HIV plasmid pNL4-3 for HIV-1 and a TA-cloned PCR fragment of beta-actin (Invitrogen, Mount Waverley, Australia). The primers and probes used were:

HIV-gag sense primer:
5'-AGTGGGGGGACATCAAGCAGCCATGCAAAT-3'
HIV-gag antisense primer:
5'-TACTAGTAGTTCCTGCTATGTCACTTCC-3'
HIV-gag detection probe:
5'-FAM-
ATCAATGAGGAAGCTGCAGAATGGGATAG-
TAMRA-3'
Beta-actin sense primer:
5'-TCACCCACACTGTGCCCATCTACGA-3'
Beta-actin anti-sense primer:
5'-CAGCGGAACCGCTCATTGCCAATGG-3'
HIV-gag detection probe:
5'-FAM-ATGCCCTCCCCCATGCCATCCTGCG-
TAMRA-3'

Proviral DNA was detected by PCR assay using the same HIV-*gag* specific primers. ICAM-1 mRNA quantification was performed by real-time PCR using primer set:

ICAM-1 sense primer:
5'-CCGAGCTCAAGTGTCTAAAG-3'
ICAM-1 antisense primer:
5'-TGCCACCAATATGGGAAGGC-3'

The reaction conditions were the same as for the amplification of HIV-*gag*, except for the addition of 10,000 times dilution of Sybr Green used instead of the flurogenic probe (Molecular Probes, Eugene, OR, USA). Product was quantified by the threshold cycle number. Amplification of product of correct size was confirmed by ethidium bromide stained gel analysis.

Histochemical analysis
Activated caspase-3 was identified using immunohistochemistry on MAGIC-5 cells grown in glass chamber slides. Cells were fixed in acetone/methanol, permeabilised with 0.025% (v/v) Triton-X 100 and blocked with 5% (v/v) normal goat serum. Cells were then stained with polyclonal caspase-3 antibody (1/400; Becton-Dickinson, Palo Alto, CA) and monoclonal vimentin antibody (1/200; Becton-Dickinson, Palo Alto, CA) for 1 hour at 37˚C. Following washing, cells were then incubated with the appropriate secondary antibodies (goat anti-mouse Alexa 488 or goat anti-rabbit Alexa 594 (Molecular Probes). Nuclear staining was performed using DAPI (1µg/ml) (Sigma-Aldrich, Castle Hill, Australia).

Methylation analyses
Genomic DNA was extracted from siRNA transfected HIV-1 infected MAGIC-5 cell cultures. Methylation of CCGG site at –146 upstream of the transcription start site was determined by restriction enzyme digest of DNA by *Hpa*II (New England BioLabs, Beverly, MA), followed by PCR amplification using a primer set of 5'-CTGAGAGAGAAGTGTTAGAGTGGAGGTTTG-3' and 5'-TTTTTTCCCATTTATCTAATTCTCC-3'. Prior to restriction enzyme digestion, the amount of input DNA was normalized to 3000 copies of HIV-1. High resolution analysis of the methylation status of CpG sites in the HIV-1 5'LTR was determined using bisulfite sequencing (Clark et al, 1994). Bisulfite-modified DNA was used in a nested PCR amplification with the first primer set:

Sense primer:
5'-YTGAGAGAGAAGTGTTAGAGTGGAGGTTTG-3'
Antisense primer:
5'-TTTTTTCCCATTTATCTAATTCTCC-3'
And, the second primer set:
Sense primer:
5'-YTGAGAGAGAAGTGTTAGAGTGGAGGTTTG-3'
Antisense primer:
5'-TCTCRCACCCATCTCTCTCCTTC-3'

PCR fragments were TA cloned and at least 10 colonies were sequenced. To confirm the methylation-related silencing effect, the methylation inhibitor, 5-azacytidine (Sigma) was added in varying concentrations to cultures 6 days after transfection with siRNA. After 30 hr, mRNA was extracted and subjected to real-time analysis as described above.

Analysis of transcriptional activity
Nuclear run-ons were performed essentially as described (Greenberg and Bender, 1997) on the nuclei isolated 3 days post transfection with siRNA. MAGIC-5 cells were infected for 3 days prior to the transfection. HIV-1 *pol* probes were synthesized by PCR amplification of HIV-1 DNA with the primer set:

pol-F:
5'-GCTTCCACAGGGATGGAAAGG-3'
pol-R:
5'-GGCTCTTGATAAATTTGATATGTCCATTGG-3'

Beta-actin probes were prepared similarly using primer set:

actin-F:
5'-TCACCCACACTGTGCCCATCTACGA-3'
actin-R:
5'-CTCCAACCGACTGCTGTCACCTTCAC-3'

Both DNA fragments were immobilized on a nylon membrane (Zeta-Probe Membrane, Bio-Rad, Hercules, CA). Hybridization was carried out for 16 hr at 42˚C using ULTRAhyb, (Ambion, Austin, TX), followed by washing with 5xSSC and 1xSSC. The membrane was scanned in a PhosphorImager (Molecular Dynamics, NJ).

RESULTS

Prolonged suppression achieved by a siRNA targeting HIV-1 promoter
We first asked if a siRNA targeted to the HIV-1 promoter could induce durable, potent suppression and transcriptional silencing of the HIV-1 provirus in cultured cells. We designed a 21 nucleotide siRNA (HIV-prom-A siRNA) with a sequence identical to a site in U3 region of the HIV-1 LTR, at ~-100 with respect to the transcriptional start site (Figure 1A). This region contains two NF-κB binding sites arranged in tandem separated by a four-nucleotide gap and is highly conserved among different HIV-1 subtypes. The nucleotide sequence within the NF-κB binding motif appears to be optimized for HIV-1 gene expression and differs from that found in the promoter regions of human genes (Chen-Park et al, 2002). The target sequence of HIV-prom-A siRNA includes the 5' NF-κB binding site, the four intervening nucleotides and the first six nucleotides of the next NF-κB binding site. A BLAST search of the human genome failed to identify sequences homologous to HIV-prom-A siRNA, with the most similar sequence sharing 17 out of 21 bases, suggesting that there are no significant endogenous targets for HIV-prom-A siRNA.

To assess the effects of HIV-prom-A siRNA, we initially used MAGIC-5 cells in which productive infection with the HIV-1 molecular clone NL4-3 was established 7 days prior to transfection with siRNA. New virus production was quantified by reverse transcriptase (RT) levels in culture supernatants. Significant levels of RT at day 6 following infection confirmed established, productive NL4-3

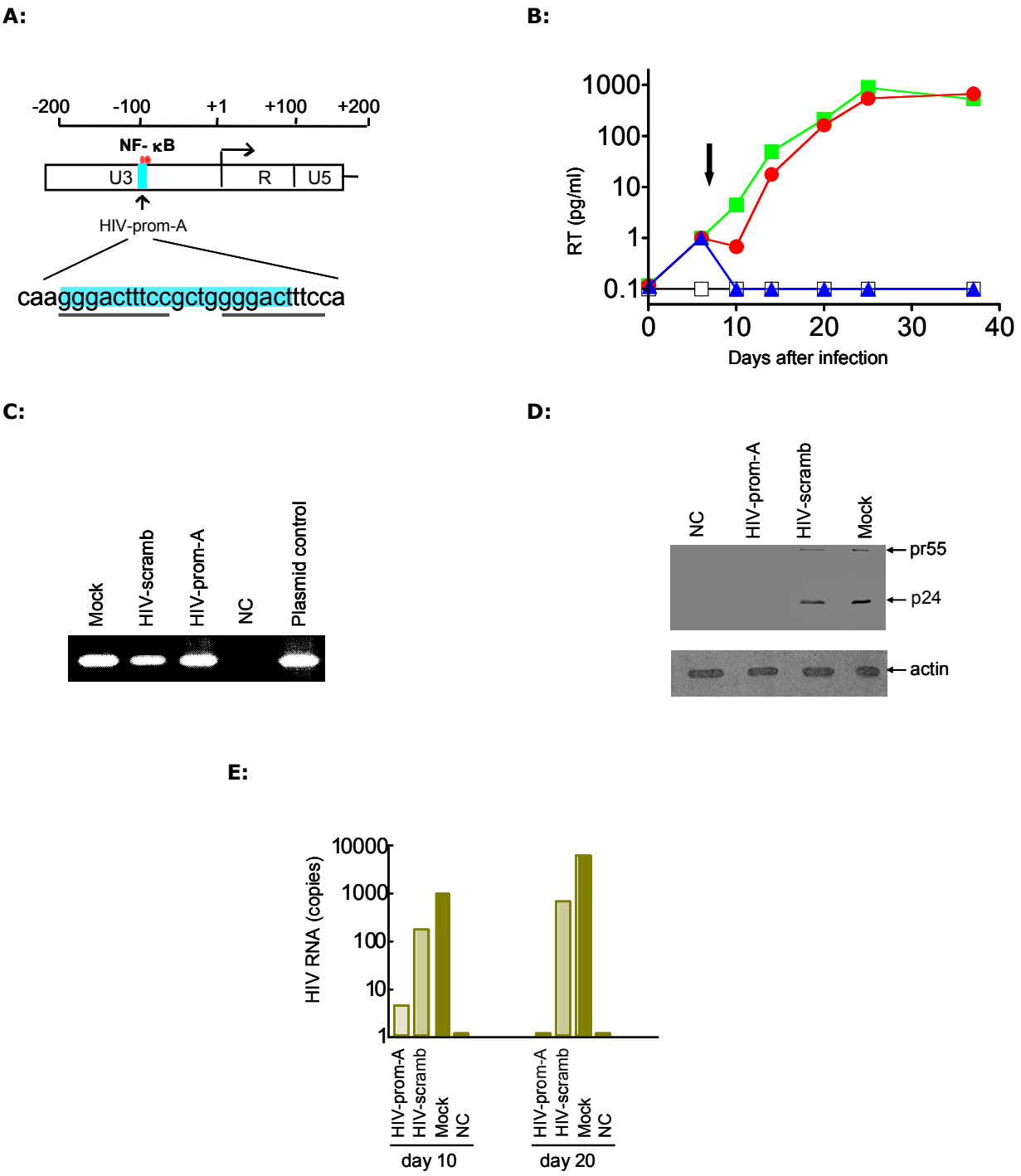


Figure 1. A siRNA targeting the HIV-1 promoter inhibits HIV-1 replication: **(A)** Location of sequence targeted by the siRNA. The positioning of NF-κB binding sites in the 5'LTR are indicated by red dots; the blue shading represents the target sequence of the siRNA (HIV-prom-A); the underlining indicates the sequence of the tandem NF-κB binding motifs. **(B)** Effect of short siRNAs on the time course of HIV-1 (NL4-3) production in MAGIC-5 cells: At day 7 siRNAs were transfected into productively infected cells (indicated by arrow). Reverse transcriptase (RT) levels are shown for cultures transfected with HIV-prom-A siRNA (blue triangle) and scrambled siRNA (red circle), mock transfected cells (green square), and uninfected cells (black square). **(C)** HIV-1 proviral DNA is present in HIV-prom-A siRNA transfected cells. Provirus was detected by PCR amplification of 154bp fragment of HIV-1 *gag* gene from DNA extracted from MAGIC-5 cells 25 days after infection. NC indicates non-infected MAGIC-5 cells. **(D)** Abrogation of HIV-1 protein synthesis induced by HIV-prom-A siRNA shown by western blot analysis of HIV-Gag-p24, Gag precursor protein p55 and beta-actin expression in cell lysates of MAGIC-5 cells 10 days after infection with NL4-3. **(E)** HIV-1 RNA is markedly reduced in the nuclei of cells transfected with HIV-prom-A siRNA. Viral RNA was quantified by real time PCR in the nuclei isolated from MAGIC-5 cells either 10 days or 20 days after infection. HIV viral RNA copy number is shown as copies per 1000 copies of beta-actin. NC indicates non-infected MAGIC-5 cells.

infection prior to transfection with siRNA. By day 20 after infection (day 13 post-transfection), viral RT levels were reduced over 1000-fold when compared to either mock transfected cultures or cultures transfected with a scrambled siRNA (Figure 1B). This suppression of productive infection was maintained for at least 30 days after a single transfection of HIV-prom-A siRNA. Proviral DNA could still be detected within cell cultures 25 days after infection (18 days after transfection), indicating that although productive infection and viral turnover were absent, the cells retained HIV-1 provirus (Figure 1C). No variation was detected in the HIV-prom-A siRNA target sequence at day 38 post infection (data not shown). Western blot analysis showed HIV-1 specific viral protein p24 and precursor p55 were markedly decreased in cultures treated with HIV-prom-A siRNA at day 10 after HIV infection (day 3 post-transfection) (Figure 1D). Real-time PCR analysis showed HIV-1 RNA levels in the nuclei isolated from MAGIC-5 cells at day 20 after HIV infection (day 13 post-transfection) were over 1000 fold lower in HIV-prom-A siRNA treated cultures compared to cultures treated with mock siRNA (Figure 1E). Although there is some apparent degree of suppression of HIV-RNA in the cultures transfected with scrambled siRNA, these, presumably non-specific effects, are approximately two orders of magnitude less than the effect of the HIV-prom-A siRNA.

The inhibition of productive HIV-1 infection was supported by a number of other observations. Productive infection with NL4-3 in MAGIC-5 cells is characterized by increasing syncytia formation, caspase-3 activation in multinuclear cells and apoptotic cell death. *In vitro*, HIV-1 infection of this cell line is characterised by elevated caspase-3 activity. We therefore monitored the effect of HIV-prom-A siRNA on caspase-3 activity as a way assessing the effects of cell associated infection. Transfection of infected cultures with HIV-prom-A siRNA was associated with reductions of each of these parameters to levels seen in non-infected cells, while levels in mock or scrambled siRNA transfected cultures appeared comparable to un-manipulated infected cultures (Figure 2). HIV-prom-A siRNA treatment did not alter the cell surface expression of proteins CD4 or CCR5, or the rate of MAGIC-5 cell proliferation (data not shown).

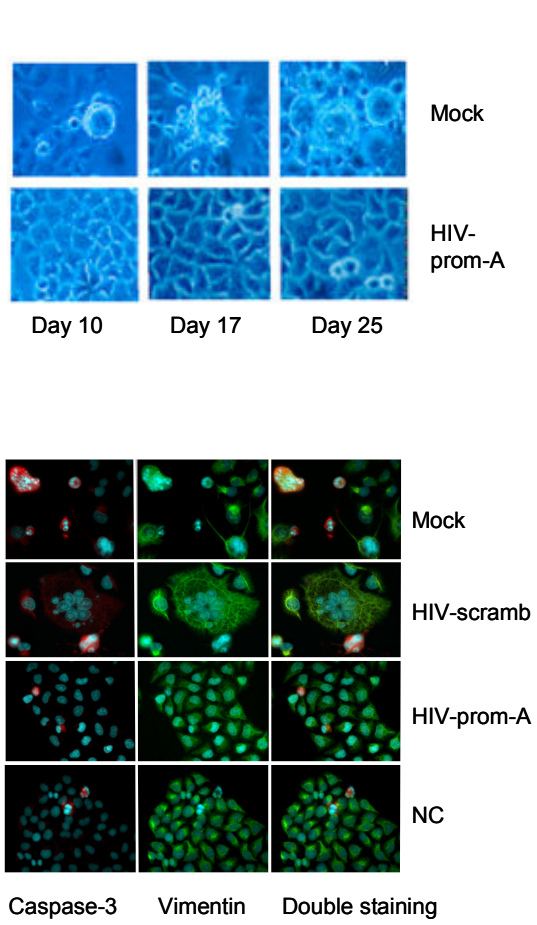


Figure 2. Prom-A siRNA inhibits syncytia formation and host cell death. **(A)** HIV-prom-A siRNA reduces syncytium formation in infected MAGIC-5 cells (Phase-contrast microscopy of MAGIC-5 cells at 200X magnification). The time points indicate days after infection (see Fig. 1B). The prominent syncytia seen in the mock-transfected cells are absent from the HIV-prom-A siRNA transfected culture. **(B)** Caspase-3 activity is reduced to normal levels in HIV-1 infected cells by transfection of HIV-prom-A siRNA. Caspase-3 activity (red) is detected in MAGIC-5 cells transfected with siRNA 7 days earlier. DAPI staining (blue) outlines the nuclei, and cell structure was highlighted by Vimentin staining (green). NC indicates non-infected MAGIC-5 cells.

Suppression of other HIV-1 isolates by HIV-prom-A siRNA

We investigated whether HIV-prom-A siRNA could induce similar effects in cultures infected with other isolates of HIV-1. The effect of this siRNA was tested on two other laboratory strains of HIV-1 (IIIB/LAI and RF) and two clinical isolates (CL-1 and CL-2) derived from patients who had failed combined anti-retroviral therapy (Suzuki et al, 2001). In these set of experiments we infected MAGIC-5 cells with HIV isolates 5 hours prior to transfection with siRNA. Under these experimental conditions, at day 7 after infection with the HIV-1 isolates NL 4-3, IIIB/LAI, RF, CL-1 and CL-2, RT activity was reduced by 900, 200, 150, 1500 and 150 fold, respectively, in MAGIC-5 cells transfected with HIV-prom-A siRNA compared to mock controls (Figure 3).

HIV-prom-A siRNA suppression is HIV-1 specific

We then checked the specificity of the suppression induced by HIV-prom-A siRNA by studying its effect on productive HIV-2 infection and expression of ICAM-1. Both have single NF-κB binding motifs in their promoter regions (Figure 4A). In HIV-2 the single NF-κB binding motif is homologous to that of HIV-1, but the flanking sequence is not, resulting in a five nucleotide difference from the HIV-prom-A siRNA sequence. The single NF-κB binding motif of ICAM-1 differs by 3 nucleotides from the NF-κB binding motif of HIV-1 and also differs in the flanking regions, resulting in a 9 nucleotide difference from HIV-prom-A siRNA. MAGIC-5 cells were infected with laboratory strain HIV-2 CBL-20, prior to being trans-

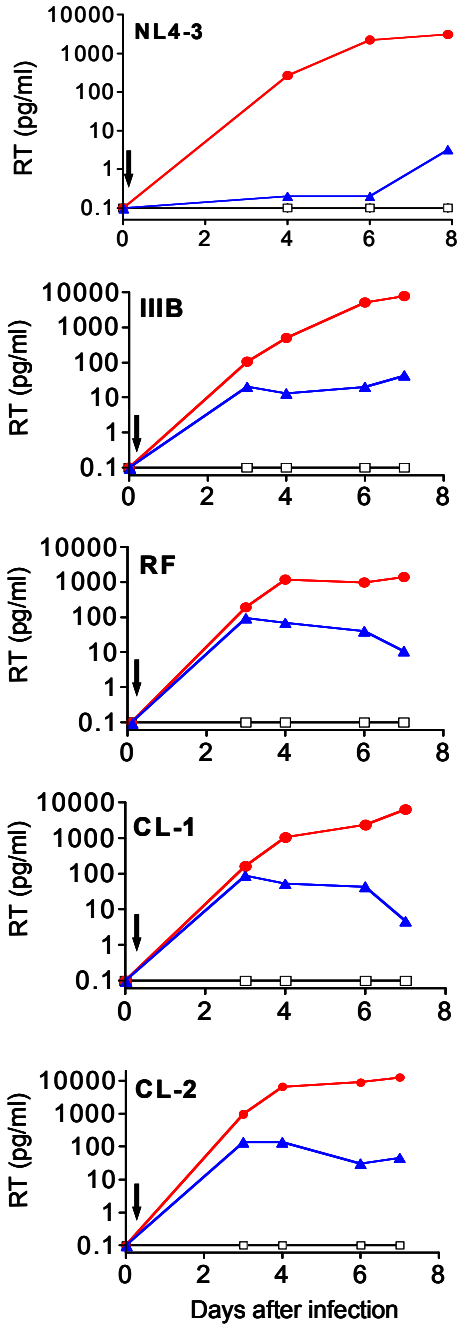


Figure 3. A siRNA inhibits replication of 5 different strains of HIV-1. Graphs show kinetics of viral replication following transfection of HIV-prom-A siRNA into MAGIC-5 cells 5 hours after infection with laboratory strains of HIV-1 (NL 4-3, IIIB/LAI, and RF) or two clinical isolates (CL-1 and CL-2) from patients failing multiple combination anti-retroviral regimens (Kaufmann et al, 2001). HIV infection was allowed to establish during a 5 hour incubation. Thereafter, cells were transfected with HIV-prom-A siRNA. Infection of MAGIC-5 cells was confirmed by HIV DNA-PCR (data not shown). Reverse transcriptase (RT) levels are shown for cultures transfected with HIV-prom-A siRNA (blue triangle), mock transfected cells (red circle), and uninfected cells (black square). Black arrow indicates timing of transfection.

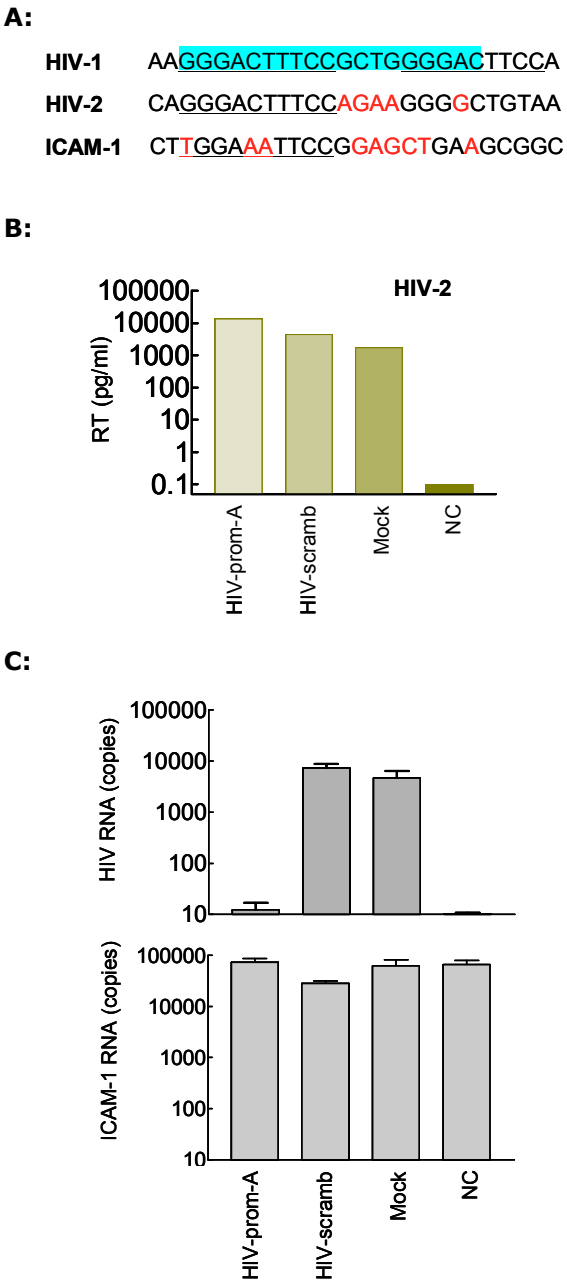


Figure 4. Specific suppression achieved after transfection of prom-A siRNA. **(A)** The promoter regions of HIV-1, HIV-2, and ICAM-1 are aligned. Sequence corresponding to HIV-prom-A siRNA highlighted in blue. Non-homologous bases in HIV-2 and ICAM-1 are indicated in red. Underlining indicates the NF-κB binding sites. **(B)** HIV-prom-A siRNA does not inhibit HIV-2 infection. Histogram shows RT levels 8 days after transfection with siRNAs, 8hours after infection of MAGIC-5 cells with laboratory stain HIV-2 CBL-20. No marked difference was observed among cultures transfected with HIV-prom-A, scrambled siRNA or mock transfection. NC indicates non-infected MAGIC-5 cells. **(C)** ICAM-1 gene expression is not affected by HIV-prom-A siRNA transfection. MAGIC-5 cells were infected with HIV-1 molecular clone NL4-3 for 5 hours followed by transfection with siRNAs. Expression levels of HIV-1 *gag* and ICAM-1 mRNA at day 3 post infection are shown in the histograms normalized to 10^3 copies of β-actin mRNA. Mean and standard deviations were obtained from three different experiments. Mock: transfection with lipofectamine only, NC: non-infected MAGIC-5 cells.

fected. HIV-prom-A siRNA did not induce any suppression of HIV-2 infection following 8 days of culture (Figure 4B). Transfection of HIV-prom-A siRNA into MAGIC-5 cells infected with NL 4-3 did not alter ICAM-1 expression despite suppressing HIV-1 infection (Figure 4C).

Transcriptional gene silencing is induced by HIV-prom-A siRNA

Nuclear run-on assays provide a direct assessment of transcription and can distinguish transcriptional from post-transcriptional effects. Run-on assays of the nuclei isolated from infected cultures 3 days after transfection of HIV-prom-A siRNA indicate that transcription of HIV-1 was fully suppressed (Figure 5). By contrast, HIV-1 transcriptional activity was evident in cultures treated with scrambled siRNA or mock transfection.

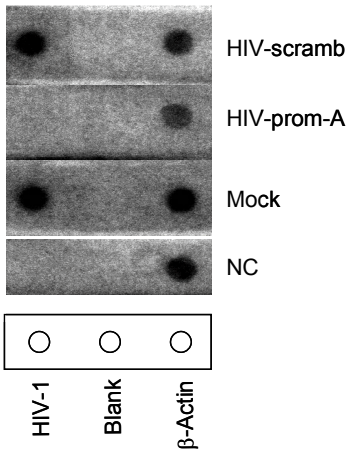


Figure 5. Nuclear run-on assays indicate transcriptional suppression by HIV-prom-A siRNA. Results of a representative assay (n = 3) in which cDNAs were immobilized on nylon membranes, and probed with RNA extracted from isolated the nuclei after run-on transcription with ^{32}P-labelled UTP (guide strip at bottom; blank: no cDNA). *De novo* transcription of HIV-1 is evident in the mock-transfected culture and the culture transfected with the scrambled siRNA. HIV-1 transcription is absent from uninfected cells (NC) and cells transfected with HIV-prom-A siRNA.

Differential levels of suppression by three other siRNAs targeting HIV-1 promoter

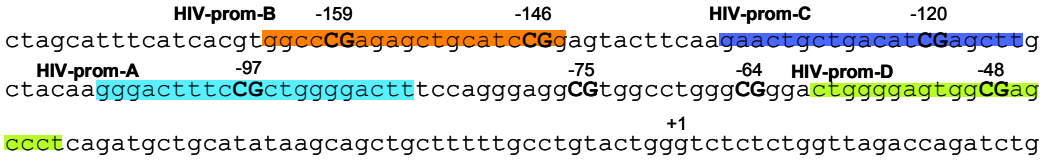
We designed three additional siRNAs (HIV-prom-B, C and D siRNAs) targeting sequences containing CpG sites in regions adjacent to that targeted by HIV-prom-A siRNA (Figure 6A) and assessed the relative efficacy of each of these constructs under the same cell culture conditions. As a further comparator we included a siRNA targeting a *gag* sequence, previously reported to be an effective inhibitor of HIV-1 replication acting by PTGS (Novina et al, 2002). The kinetics of viral suppression varied among the siRNAs tested (Figure 6B). By day 18 after infection (day 11 post-transfection) RT levels were reduced over 1000-fold in the cultures transfected with either HIV-prom-A or B siRNAs, when compared to mock transfected cultures. This degree of suppression was maintained until at least day 38 after infection (day 31 post-transfection) with HIV-prom-A siRNA, whereas there was evidence of some viral production by day 38 with HIV-prom-B siRNA. HIV-prom-C siRNA was able to suppress viral production transiently until day 14 after infection (day 7 post transfection) and HIV-prom-D siRNA did not induce any significant viral suppression. HIV-*gag* siRNA, suppressed viral production until day 14 after infection (day 7 post transfection), but thereafter viral production approached levels seen in mock transfected cultures. The extent of inhibition of HIV-1 replication induced by this siRNA in these experiments was similar in magnitude and duration to that previously reported (Novina et al, 2002). Cell associated proviral HIV-1 DNA was detectable up to and including day 38 in all cultures (Figure 6C).

RdDM is induced by siRNAs targeting HIV-1 promoter

The suppression of HIV-1 induced by HIV-prom siRNAs could have occurred through either a transcriptional (RdDM) or a post-transcriptional (RNAi) mechanism, but persistence of the effect and the run on data suggested to us that these siRNAs may have induced transcriptional silencing through RdDM. To investigate methylation of CpGs in the HIV-1 LTR, we treated DNA, extracted from cultures at 14, 21, and 38 days after infection (days 7, 14, and 31 post-transfection respectively) with the methylation sensitive restriction enzyme *Hpa*II (Figure 6A and 7A). A PCR amplicon of the HIV-1 5'-LTR was generated from *Hpa*II-digested DNA derived from HIV-prom-A, B, and D siRNA-treated cells, indicating methylation of CpGs at the *Hpa*II site at each of the time points sampled (Figure 7A). On the other hand, there was no evidence of methylation at this site following transfection with scrambled, *gag* or HIV-prom-C siRNA constructs or in mock transfected cultures. PCR amplification of this region prior to digestion by *Hpa*II generated bands in all infected cultures regardless of treatment confirming the presence of HIV DNA in these cells at all time points, independently confirming the data regarding the presence of provirus generated by PCRs of the *gag* region (Figure 6C).

To explore the induction of methylation by HIV-prom siRNAs in more depth, we sequenced bisulfite-treated genomic DNA extracted from the MAGIC-5 cultures 38 days after initial transfection (Figure 7B). Transfection of HIV-prom-A siRNA was associated with cytosine DNA methylation at 6 of 7 CpGs sites studied within the LTR, including the CpG site in the target sequence (position –97 relative to the transcription initiation site of the TAR region; Figure 5A) in 10/10 clones. Transfection of HIV-prom-B siRNA was associated with DNA methylation at 5 of 7 CpG sites in at least 8/10 clones at each site. HIV-prom-C and HIV-prom-D siRNAs induced less intense methylation at fewer sites (Figure 7B). No significant CpG methylation was seen in mock, HIV-*gag* siRNA or scrambled siRNA transfected cultures. In all cases, including HIV-prom-C, the methylation status at the CpG site at –146, as determined by *Hpa*II digestion was consistent with that demonstrated by bisulfite sequencing. It is notable that all four promoter-targeted siRNAs induced DNA methylation at

A:



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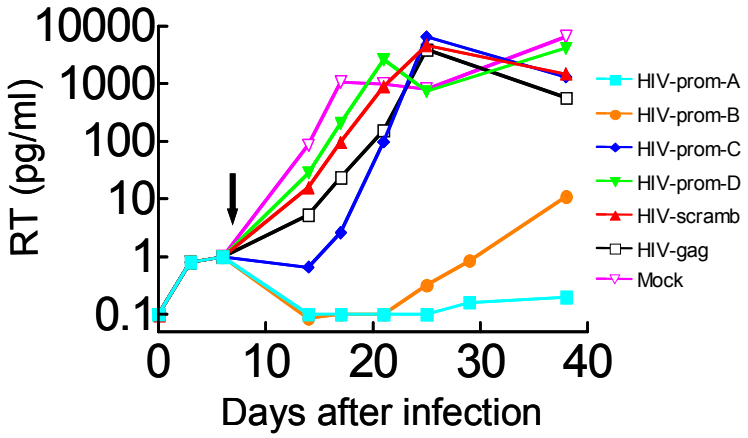

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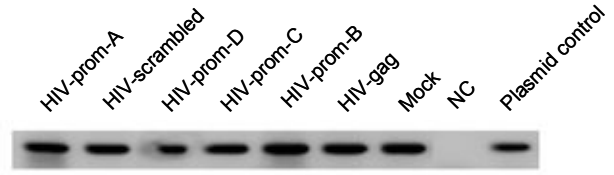
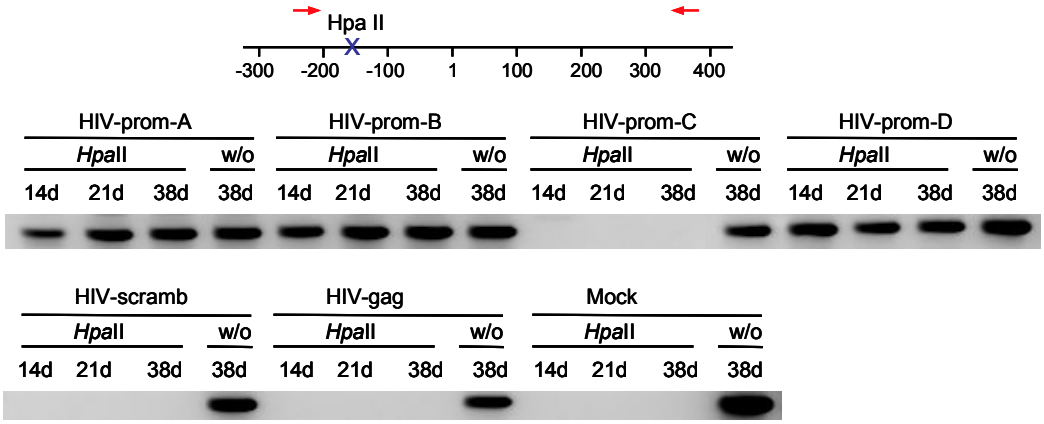


Figure 6. Effect of six short siRNAs on productive HIV-1 infection including 4 siRNAs targeting HIV-1 promoter region and siRNA HIV-*gag*, which is known act as PTGS. **(A)** The sequences within HIV-1 5'LTR targeted by HIV-prom-A-D siRNAs are indicated by highlighting. HIV-prom-B siRNA target sequence includes CpG sites at –159 and –146, HIV-prom-C siRNA target sequence includes CpG at –120, and HIV-prom-D siRNA target sequence includes CpG at –48. CpGs within the 5'LTR of HIV-1 are indicated by emboldened text. Nucleotide numbering is relative to the transcription start site and sequence is that of HIV-1 strain NL4-3. Each siRNA contains at least one CpG site. **(B)** Effect of siRNAs on the time course of HIV-1 (NL4-3) production in MAGIC-5 cells. At day 7 post-infection siRNAs were transfected into the cells (indicated by arrow). Reverse transcriptase (RT) levels are shown for cultures transfected with HIV-prom-A, B, C, D, HIV-1-gag, and scrambled siRNAs as indicated in the figure legend. **(C)** HIV-1 proviral DNA is present in cultures transfected with HIV-prom siRNAs regardless of whether infection is productive or not. PCR amplification of proviral DNA extracted from MAGIC-5 cells 38 days after infection (Figure 6B). The amplified region is a 154bp fragment of the HIV-1 *gag* gene. NC indicates non-infected MAGIC-5 cells.

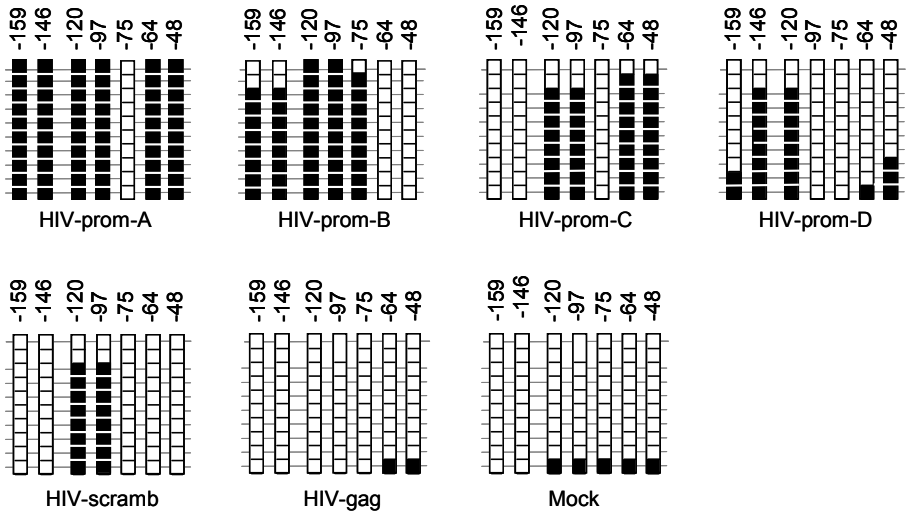
the CpG sites contained within their target sequences. Furthermore, bisulfite sequencing revealed the same pattern of CpG methylation at day 3 post-transfection in cultures treated with HIV-prom-A siRNA, suggesting the methylation patterns seen are established early and are then maintained (data not shown).

To further explore the link between cytosine methylation and the reduction in virus production, we treated cells with the methylation inhibitor 5-azacytidine (5-aza-C), and then determined intracellular viral RNA levels by RT-PCR. Treatment with 5-aza-C for 30 hours partially reversed the suppression induced by HIV-prom-A siRNA, increasing HIV-RNA production by 2-25 fold (Figure 6D). These data indicate increased viral turnover is related to chemical inhibition of DNA methylation.

A:



B:



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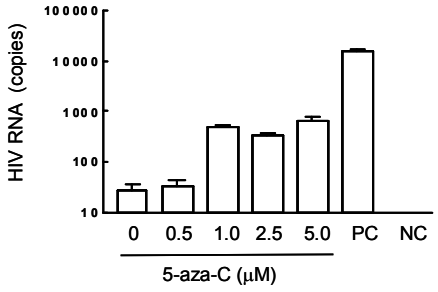

Figure 7. HIV-prom-A siRNA induces CpG methylation of the HIV-1 LTR and transcriptional suppression. **(A)** The DNA from HIV-1 provirus in cells transfected with HIV-prom-A, B, and D siRNA is protected from digestion with the methylation-sensitive restriction enzyme *Hpa*II. DNA was extracted from HIV-1 infected cells at 14, 21, and 38 day after infection (day 7, 14, and 31 post-transfection respectively), digested with *Hpa*II and subjected to PCR using primers with binding sites denoted by arrows above the map. Nucleotide numbering is relative to the transcription initiation site. The downstream primer is located outside the LTR, so that this primer set specifically amplifies only the 5' LTR. The restriction enzyme *Hpa*II is methylation sensitive such that it will not cut if the CpG in the CCGG recognition site is methylated. *Hpa*II cuts this region of HIV-1 at the single CpG site at position -146 (labeled *Hpa*II); PCR amplification will occur only if the DNA between the primer binding sites has not been cut. W/O indicates that DNA has not been subjected to *Hpa*II digestion. Amplification of proviral DNA from mock-transfected cells

was abrogated by pre-digestion with *Hpa*II, indicating that it was unmethylated. By contrast amplification from *Hpa*II-digested DNA is successful from the cultures transfected with HIV-prom-A, B, and D siRNA, indicating that the *Hpa*II sites were methylated. **(B)** Bisulfite allelic sequencing of the 5' LTR of the HIV-1 provirus at 38 day after infection. Bisulfite-modified genomic DNA was PCR amplified with nested primers specific for the bisulfite-converted sequence (see Methods) and ligated into a plasmid vector; at least 10 individual plasmid clones were sequenced. Methylated cytosines are spared from bisulfite conversion, while unmethylated cytosines are converted to uracils. The figure shows sequences of individual clones as horizontal lines, with the CpGs numbered as in Figure 6A; unfilled boxes are unmethylated CpGs, and filled boxes are methylated CpGs. **(C)** The demethylating agent 5-azacytidine (5-aza-C) partially restores HIV transcription in cells previously transfected with HIV-prom-A siRNA. Real-time PCR amplification of the *gag* region from RNA was used to detect productive infection in MAGIC-5 cells. Six days after transfection with siRNA cells were treated with 5-aza-C at the indicated concentrations for 30 hours. The positive control (PC) was a culture in which no siRNA was transfected, and the negative control (NC) was uninfected cells. The results are shown as HIV RNA copy number per 1000 copies of beta-actin.

DISCUSSION

Taken together, these findings derived from four different siRNAs targeting HIV-1 promoter region, suggest that these duplexes induce RdDM of the 5'LTR of HIV-1 to varying extents, both in terms of number of CpG sites involved and the density of the methylation that occurs at each site. The degree and density of methylation in turn correlates with the effectiveness of viral suppression. Methylation includes the CpG sites within the sequence homologous to the siRNA but extends to adjacent CpG sites. Spreading of DNA methylation to adjacent CpG sites has been observed previously, most commonly in plants (Jones et al, 1999; Van Houdt et al, 2003; Xie et al, 2004; Wang and Metzlaff, 2005), but also in mammalian cells (Kawasaki and Taira, 2004). The time course of suppression contrasts markedly with transient suppression of HIV-1 production induced by transfection of siRNAs which target HIV-1 structural or regulatory genes and act by PTGS (Capodici et al, 2002; Novina et al, 2002).

Although some similarities exist between plants and mammals in the enzyme systems involved in the maintenance of DNA methylation at CpG sites (Cao et al, 2003; Bender, 2004), recent publications suggest that an enzyme essential for de novo RNA-directed DNA methylation in plants, a subunit of RNA polymerase, is not found in mammalian cells, but is unique to plants and there is no clear homologue in mammalian cells (Kanno et al, 2005). However, current reports demonstrate that DNA methylation can be induced by siRNA in mammalian cells (Kawasaki and Taira, 2004; Morris et al, 2004; Castanotto et al, 2005). Clearly, the underlying pathways involved in this process still require further elucidation.

In the case of the most effective of the constructs tested here, CpG methylation is induced early and then maintained over the period of observation. The potency and persistence of the effect is related to the degree and pattern of CpG methylation induced. The onset of the effect is rapid as indicated by the early suppression of viral protein and RNA expression, the early inhibition of transcription as demonstrated by the run on assays and the early onset of *de novo* methylation as demonstrated by the *Hpa*II digests. The constructs clearly vary in their potency, but the determinants of potency are still to be elucidated. The effect is specific, it is abrogated by variation in the target sequence, but is maintained if the target sequence is highly homologous to the duplex transfected. This observation is similar to, and extends, the well described ability of siRNAs to induce prolonged TGS in plant systems (Wassenegger, 2000) and supports some recently published data regarding this phenomenon in mammalian cells (Kawasaki and Taira, 2004; Morris et al, 2004). However, in both these later reports the silencing was relatively short-lived (up to 8 days) even when multiple siRNAs were used in combination (Kawasaki and Taira, 2004). In our hands HIV-prom-A and -B siRNAs induced prolonged suppression of themselves, in the absence of any requirement for combination.

The stability of suppression induced by HIV-prom-A siRNA may result from methylation of the LTR and inhibition of transcription. Although this mechanism is well described in plants and similar observations have been made in centromeric chromatin in yeast, the precise molecular events producing the effect are not well understood. It remains unclear how these promoter targeted siRNA induce CpG methylation and that how is this maintained and extended beyond the target sequence. However, hints as to the possible mechanism can be derived from observations in plants, yeast and mammalian cells. Firstly, it is known that the induction of methylation at CpG sites in targeted promoter regions after transfection of siRNA is dependent upon DNMT (Kawasaki and Taira, 2004). More recently it has been hypothesized that in yeast initiation and extension of *de novo* CpG methylation is dependent upon the RITS complex. This complex consists of both dicer derived siRNA and a range of proteins including Chp1, Tas3 and Ago1 (Schramke and Allshire, 2003; Noma et al, 2004; Verdel et al, 2004). All these proteins are associated with silenced centromeric heterochomatin in yeast. Furthermore, once binding is initiated RITS complexes can act on adjacent sequences. The stability of RITS association with heterochomatin appears to be dependent upon Histone 3-Lys-9 methylation and this is critical for its role in TGS (Rea et al, 2000; Zilberman et al, 2003; Kawasaki and Taira, 2004). Importantly, a large number of the components of the RITS complex and its associated machinery seem to be identical to those used by the RISC (RNA-induced silenced complex) in the process of siRNA induced PTGS (Motamedi et al, 2004; Noma et al, 2004; Verdel et al, 2004). The precise relationships between the actions of promoter targeted siRNA, the RITS complexes, DNA compaction and DNA methylation in the promoter region still require further elucidation.

The process of TGS by the RITS complex is dependent upon an effective nucleation site (Noma et al, 2004). This site is determined by sequence homology with the siRNA

included in the RITS complex. It is conceivable that silencing efficiency will depend on the ability of the targeted sequence to act as an effective nucleation site. Therefore, the efficiency of HIV-prom-A and -B siRNAs to suppress gene expression relative to those siRNAs targeting other promoter regions may reflect inherent, as yet to be described differences in targeted promoter regions.

Further support for CpG methylation of the 5'LTR being a plausible component of transcriptional gene silencing of HIV-1 comes from the literature concerned with retroviral latency. The 5'LTR of HIV-1 plays a critical role in the regulation of proviral transcription containing sites that bind a range of viral and cellular transcription factors including NF-κB (Nabel and Baltimore, 1987; Perkins et al, 1993; Okamoto et al, 1996). Previous studies have demonstrated that methylation of CpG sites within the 5'LTR is associated with inhibition of viral transcription (Gutekunst et al, 1993). HIV-1 latency in transgenic murine models is associated with CpG methylation within the 5'LTR (Tanaka et al, 2003). Conversely, demethylation of these sites and histone acetylation within the 5' LTR region is associated with viral activation (Lusic et al, 2003). Furthermore, true viral latency of the related retrovirus, human T-cell leukemia virus type 1 (HTLV-1), is associated with methylation of CpG sites within the 5'LTR of that virus (Koiwa et al, 2002). Thus RdDM may provide a mechanism by which latency of the HIV-1 provirus can be induced. The continued presence of proviral DNA in the absence of productive infection in the cultures transfected with HIV-prom-A siRNA, along with the methylation data suggest that these siRNAs are inducing a state akin to viral latency.

While targeting HIV-1 structural or accessory genes by siRNA induces rapid degradation of viral RNA, the duration of suppression is short lived (Capodici et al, 2002). The duration of this effect can be extended up to 14 - 25 days using recombinant adeno-associated or lentiviral vectors to deliver siRNA. However, these approaches still compromised by the ability of the virus to evolve as a result of changes in environmental pressures. Viral evasion of this type of suppression may also be mediated by non-mutational mechanisms. Recently, Tat has been shown to act as a suppressor of dicer activity, inhibiting the processing of shRNA into iRNAs (Bennasser et al, 2005). Another possible mechanism of RNA induced suppression of HIV replication has recently been described. Single stranded microRNA, derived from HIV-*nef* sequences found in long term non-progresses, can induce short term (48h) translational repression of Nef expression (Omoto et al, 2004).

The advantages of RdDM over a mechanism that degrades RNA after transcription are not limited the half-life of its effect. The high mutation rate of HIV-1, stemming from its non-proof reading reverse transcriptase and high replication rates, confers an ability to adapt to environmental pressures, including anti-retroviral drugs and immune responses (Miller and Larder, 2001; Klenerman et al, 2002), and this can result in eventual loss of efficacy of an RNAi inducing siRNA (Boden et al, 2003). Transcriptional suppression by RdDM reduces viral turnover markedly, and if no new copies of RNA are being made, then the viral RT has no substrate upon which to generate mutants that may have a survival advantage. We have tried to exploit this advantage further by targeting with HIV-prom-A, a highly conserved region of HIV-1, the nucleotide sequence of which appears to have been optimized for efficient gene expression (Chen-Park et al, 2002). In addition to suggesting a therapeutic strategy for HIV-1 infection, our findings demonstrate that a pathway of RNA-directed silencing and methylation exists in mammalian cells. This observation and recently published data strongly suggest that, as it has been shown to do in yeast and plants (Hall et al, 2002; Verdel et al, 2004), the pathway may maintain silent chromatin in vertebrates (Volpe et al, 2002), and may be amenable to the induction of stable silencing of other sequences in mammals.

CONCLUSIONS

- siRNAs targeting the promoter region of HIV-1 can induced suppression of viral replication, cytosine methylation and transcriptional gene silencing.
- The suppression can be durable and the extent of suppression appears to correlate with the density of *de novo* cytosine methylation.
- Although the precise mechanisms involved are unclear, DNA methylation appears critical as demethylation results in partial reversal of suppression.

ACKNOWLEDGEMENTS

HIV-1 subtype B strain NL4-3, HIV-1 IIIB/LAI, RF, and HIV-2 CBL-20 were obtained from NIH AIDS Research and Reference Reagent Program catalogue numbers 114, 398, 2803, and 600. Drs Oka and Tastumi kindly provided MAGIC-5 cells. Thanks to Drs Watanabe and Ishida for informative advice about run-on assays. The National Centre in HIV Epidemiology and Clinical Research is supported by the Commonwealth Department of Health and Ageing through the Australian National Council on AIDS, Hepatitis C and Related Diseases.

STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

LIST OF ABBREVIATIONS

RITS: RNA-induced initiation of transcriptional gene silencing
RISC: RNA-induced silencing complex
RdDM: RNA-directed DNA methylation
TGS: Transcriptional Gene Silencing
PTGS: Post Transcriptional Gene Silencing

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