Novel Pol II Fusion Promoter Directs Human Immunodeficiency Virus Type 1-Inducible Coexpression of a Short Hairpin RNA and Protein

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We demonstrate a novel approach for coexpression of a short hairpin RNA (shRNA) with an open reading frame which exploits transcriptional read-through of a minimal polyadenylation signal from a Pol II promoter. We first observed efficient inducible expression of enhanced green fluorescent protein along with an anti-rev shRNA. We took advantage of this observation to test coexpression of the transdominant negative mutant (humanized) of human immunodeficiency type 1 (HIV-1) Rev (huRevM10) along with an anti-rev shRNA via an HIV-1-inducible fusion promoter. The coexpression of the shRNA and transdominant protein resulted in potent, long-term inhibition of HIV-1 gene expression and suppression of shRNA-resistant mutants. This dual expression system has broad-based potential for other shRNA applications, such as cases where simultaneous knockdown of mutant and wild-type transcripts must be accompanied by replacement of the wild-type protein.

Regulation of gene expression is governed not only by transcription initiation at the promoter but also by the polyadenylation signal sequence. A functional polyadenylation signal is required for transcription termination by RNA polymerase II; nuclear to cytoplasmic transport of the message is dependent upon polyadenylation and splicing, and these processes are coupled through the C-terminal domain of RNA polymerase (9). Small changes in overall RNA processing efficiency in a particular cell or the effective strength of a particular splicing or polyadenylation site can serve as an important control point for gene expression in a tissue- or developmental stage-specific manner (9).

A number of complex transcription units employ multiple poly(A) sequences to generate diversity from a single transcription unit (21). In some cases use of alternate poly(A) sequences can generate mRNAs of different stabilities, which could impact their translation and overall gene expression (25). In some viruses, differential strengths of the poly(A) sequence allow temporal regulation of gene expression. Polyadenylation has been shown to regulate L1 versus L3 mRNA production in adenovirus infection (7, 8). The promoter-proximal L1 poly(A) site is weaker than the promoter-distal L3 poly(A) site (29). Similarly, in the case of human papilloma virus a weaker early polyadenylation signal sequence allows significant levels of read-through to allow late gene expression during keratinocyte differentiation (35). In the present study we have employed a similar approach to coexpress two transcripts from a single transcription unit. We have exploited a weaker poly(A) signal sequence used for Pol II-based short hairpin RNA (shRNA) expression to allow read-through of a downstream protein coding sequence, thereby generating both a functional small interfering RNA (siRNA) and a translated protein from the same promoter. The rationale behind the approach is that

transcripts terminating at the minimal poly(A) sequence get processed to siRNA-sized products while the read-through of the mpoly(A) would produce an mRNA that can be translated.

Introduction of double-stranded RNA into an organism can cause specific interference of gene expression (32). The proteins mediating RNA interference (RNAi) are part of an evolutionarily conserved cellular pathway that processes endogenous cellular RNAs to silence developmentally important genes (16, 18). RNAi-mediated gene silencing in mammalian cells has been achieved either by transfecting synthetic double-stranded RNA (5, 10) plasmids expressing siRNA as individual sense and antisense strands (4, 20, 26) or by using shRNAs 21 to 29 nucleotides long that act as substrates for the enzyme Dicer and can be processed to siRNA-sized molecules that guide the cleavage of cognate mRNAs (1, 41).

We have previously reported on the development of a human immunodeficiency virus (HIV)-inducible promoter system for expressing anti-HIV shRNAs (36). In that study as in a Pol II-based shRNA expression system described earlier, a minimal polyadenylation signal sequence was used to terminate shRNA transcripts (41). Using this approach, we have observed around 80 to 90% inhibition of HIV-1 replication in CEM T cells and CD34⁺ hematopoietic progenitor cells. However, it is clear that HIV-1-resistant mutants arise quite readily under the selective pressure of siRNAs (6, 37). Thus, it makes sense to utilize a combination of siRNAs and other antivirals to achieve long-term knockdown in the absence of viral escape mutants. Since one of the restrictions for expression of a functional shRNA from Pol II promoters is the use of a minimal polyadenylation signal sequence to terminate the shRNA transcripts (41), we exploited the transcriptional read-through of a weak poly(A) signal as a means for coexpressing an shRNA and the antiviral transdominant RevM10 protein. Rev is required for nuclear to cytoplasmic transport of singly spliced and unspliced full-length genomic RNAs; therefore, it is an attractive target for HIV-1 therapy. Transdominant Rev proteins such as M10 have amino acid substitutions in the nuclear export signal of HIV-1 Rev (24) and at least one M10 variant

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has entered phase I and phase II clinical trials for AIDS gene therapy (31, 39). It is clearly demonstrable that expression of RevM10 can efficiently inhibit HIV-1 replication in T-lymphoblastoid cell lines as well as primary T cells (2, 3, 11, 27). In the present study we demonstrate efficient transcriptional read-through of a minimal poly(A) signal located downstream of an anti-*rev* shRNA, resulting in functional expression of two different proteins, an enhanced green fluorescent protein (EGFP) reporter gene or a humanized version of RevM10 (huRevM10). Moreover, the expression of both the shRNA and downstream proteins is completely dependent on HIV-1 Tat supplied in *trans*. When the shRNA and RevM10 are coinduced, there is near complete inhibition of HIV-1 p24 antigen production in stably transduced human hematopoietic cells and no shRNA-resistant virus emergence during 70 days of challenge. In contrast, a resistant mutation arose within a 40-day time frame when the anti-*rev* hairpin was constitutively expressed independent of the huRevM10.

MATERIALS AND METHODS

Constructs. Construction and characteristics of the lentiviral vector pHIV-7 have been previously described (14). The siRNA was designed to target a highly accessible site in the HIV-1 *rev* transcript as previously reported (20). For U6ShRNA, the shRNA was placed under transcriptional control of the human U6 promoter cloned in the lentiviral vector pHIV-7. Construction and characteristics of the long terminal repeat (LTR)-hsp70 promoter have been previously reported (36). In short, the exons and glucocorticoid response elements upstream of the minimal *Drosophila* hsp70 promoter component were removed from the pIND vector (Invitrogen) and replaced with the HIV-1 LTR up to and including the transactivation response element. The shRNA design involved extending the sense strand by 8 nucleotides on the 3' end to obtain a 28-nucleotide stem. A single point mutation of C to U was designed in the center of the sense strand to facilitate cloning. The sequence of the 9-base loop was as described previously (36, 41). The sequence of the minimal polyadenylation sequence described by Xia et al. (41) and used in our earlier study (36, 41) as well as in current studies is CTAGTAATAAAGGATCCTTTATCTTCATTGGATCCGTGTGTGGTTTTTGTGTGCGGCCGCTAGACC.

For LTRsh-GFPpA, EGFP-SV40poly(A) (where SV40 is simian virus 40) was PCR amplified from pEGFP-N1 (BD Clontech) and cloned downstream of the mpoly(A) signal sequence of the LTRsh-shRNA in pCR2.1 (Invitrogen) (see Fig. 2A). For the HIV-7-LTRsh-RevM10 construct, huRevM10 was cloned downstream of the mpoly(A) signal sequence of the LTRsh-shRNA, and the resulting fragment was cloned in the lentiviral vector pHIV-7 (see Fig. 2A).

Cell culture. HT1080 and 293 cells were maintained in Dulbecco's modified Eagle's medium–20% fetal bovine serum (FBS). Twenty-four hours before transfection, cells were replated in six-well plates at 50 to 70% confluence with fresh medium without antibiotics. The human T cell line CEM was maintained in RPMI 1640 medium supplemented with 10% FBS. For cotransfection, the target pNL4-3 was used in a 1:2 (wt/wt) ratio with the shRNA constructs, and transfection was carried out using Lipofectamine Plus (GibcoBRL) according to the manufacturer's instructions. For cells transfected with LTRsh-GFPpA, 72 h posttransfection, the transfected cells were visualized by fluorescence microscopy. Images were collected using an Olympus BX50 microscope and DEI-750 video camera (Optronics) at a magnification of $\times 40$ with an exposure time of 1/4 s. Induction of EGFP by pNL4-3 was confirmed in at least three independent experiments.

Northern blotting. For Northern blot analysis of shRNA expression, total RNA was extracted using RNA STAT-60 (TEL-TEST "B") according to the manufacturer's instructions. Twenty-five micrograms of total RNA was electrophoresed in a 10% polyacrylamide–8 M urea gel. RNA was transferred by electroblotting onto a Hybond-N⁺ membrane (Amersham Pharmacia Biotech). The hybridization was performed using an oligonucleotide probe complementary to the antisense strand of the siRNA. Hybridizations were carried out at 37°C, and the filters were washed twice with $2\times$ SSPE ($1\times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]) at 39°C and then once with $1\times$ SSPE at 41°C prior to autoradiographic exposure. For Northern blot analysis of RevM10 expression, a 1% agarose-formamide gel was used. RNA was transferred onto Hybond-N⁺ (Amersham Pharmacia Biotech). The hybridization

probe was prepared by random priming. The hybridization and wash steps were performed at 37°C. Human U6 snRNA or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were probed as internal standards.

Lentiviral vector production. The packaging system used was described previously (14). The lentiviral vector, pHIV-7-GFP contains a hybrid 5' LTR in which the U3 region is replaced with the cytomegalovirus (CMV) promoter, the packaging signal (Ψ), the Rev-responsive element (RRE) sequence, the EGFP gene driven by the CMV promoter, and the 3' LTR in which the *cis* regulatory sequences are completely removed from the U3 region. Thus, upon integration, transcription can only initiate at the internal promoters. Plasmids used for packaging include pCHGP-2, which codes for the HIV-1 *gag* and *pol* genes under the control of the CMV promoter. The plasmid pCMV-Rev contains the coding sequence of Rev driven by the CMV promoter. pCMV-G contains the vesicular stomatitis virus G protein gene under the control of the CMV promoter.

293T cells were cultured until they reached 80% confluence in a 100-mm culture dish. Fifteen micrograms of lentiviral vector with the appropriate insert, 15 μ g of pCHGP-2, 5 μ g of pCMV-G, and 10 μ g of pCMV-Rev were cotransfected into 293T cells using the calcium phosphate precipitation procedure (15). The packaging system is shown in Fig. 5A (14). Six hours after transfection, the culture medium was replaced. The culture supernatants were collected at 24 h and 36 h after transfection. The supernatants were pooled together, passed through a 0.45- μ m-pore-size filter, concentrated by ultracentrifugation, and stored at -80°C until use. Vector titers were determined by transduction of HT1080 cells and assayed for EGFP expression using flow cytometry. The vectors were free of replication-competent lentivirus as determined by both reverse transcription-PCR (RT-PCR) and p24 antigen assays.

Viral RNA sequencing. For sequencing viral RNA, viral RNA was extracted from the culture supernatant using a QIAamp viral RNA mini kit (QIAGEN) according to the manufacturer's instructions. The viral RNA was PCR amplified using primers flanking the target site, and the PCR product was sequenced in the City of Hope sequencing core facility.

Transduction of CEM cells. A total of 2×10^5 cells were placed in a 15-ml centrifuge tube with 1 ml of culture medium in the presence of lentiviral vector at a multiplicity of infection (MOI) of 10 and 8 μ g/ml polybrene. Following centrifugation at 2,000 rpm for 1 h, the cells were transferred into a 24-well culture plate, and after 24 h the culture medium was replaced. Seventy-two hours posttransduction, the cells were sorted for GFP expression and used for HIV-1 challenge experiments.

Primer extension analysis. For primer extension analysis, a primer flanking the AUG of the huRevM10 protein was used. The sequence of the 3' primer used is 5'-CGTGCGGCCGCGCCATGG. Total RNA was reverse transcribed using Thermoscript RNase H Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. HIV-7-LTRsh-RevM10 was simultaneously sequenced with the same primer using the Sequenase Version 2.0 DNA sequencing kit (USB Corp.) according to the manufacturer's instructions. The sequencing reaction was run along with the primer extension reaction on a 7 M urea–6% polyacrylamide gel.

HIV-1 challenge. Twenty-four hours after sorting, 1×10^6 CEM T cells were infected with HIV-1 strain IIIB at an MOI of 0.01. After overnight incubation, the cells were washed three times with Hanks' balanced salts solution and cultured in medium with R10 (RPMI 1640 plus 10% FBS). At designated time points from day 7 to day 21, 500 μ l of culture supernatant was withdrawn for analysis of the cell count. The cells were then pelleted and returned to the infection. The culture supernatant was used for a p24 assay. This was replaced with 500 μ l of fresh medium. On day 25, due to extensive cell death observed in cells transduced with vector alone, an additional 250,000 CEM vector-alone transduced cells were added to the vector-alone infection. The medium was then changed once every 5 days: on day 30 when the sample was collected for analysis, day 35, day 40 (sample collected), day 45, day 50 (sample collected), day 55, day 60 (sample collected), day 65, and day 70 (sample collected). The ratio of live cells to total cells was expressed as a survival plot.

HIV-1 antiviral assays. Cells were cotransfected with HIV-1 pNL4-3 proviral DNA and the appropriate shRNA constructs. Culture supernatants were collected at 24-h intervals and analyzed for HIV-1 p24 antigen using an enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter Corp.). The p24 values were calculated using a Dynatech MR5000 ELISA plate reader (Dynatech Lab, Inc). Cell viability was also determined using a trypan blue dye exclusion count.

RESULTS

HIV-1 escapes from RNAi. shRNAs targeted against HIV-1 genes can efficiently downregulate HIV-1 gene expression in

cells, resulting in potent inhibition of viral replication. However, earlier reports have demonstrated that viral mutants emerge during the course of challenge experiments that can escape RNAi. To investigate whether our *rev* target site could also mutate and generate a resistant variant, we cloned a U6 promoter-driven shRNA targeting a previously characterized site in *rev* (20, 36). The Pol III shRNA gene was inserted in the HIV-7 lentiviral vector (Fig. 1A). CEM T cells transduced with the HIV-7-U6SIshRNA construct were then challenged with the IIIB strain of HIV-1. At different time points viral RNA was isolated from the culture supernatant, subjected to RT-PCR amplification, and sequenced. The viral RNA isolated on day 40 showed an approximately 30% frequency of a C-to-A transversion in the target site. The supernatant from this challenge was used to infect a new population of U6SIshRNA-transduced CEM T cells. On day 15 a further enrichment of the same mutation was observed, demonstrating that under the selective pressure of RNAi viral mutants arise that can escape RNAi (Fig. 1B). The mutation is located at position 10 of the target relative to the 5' end of the siRNA targeting this site, a position in which mutations are known to severely decrease cleavage activity (30). A p24 ELISA of the culture supernatant confirmed efficient replication of the mutant virus in both the vector-transduced as well as U6SIshRNA-transduced cells (Fig. 1C). Northern blot analyses of total RNA extracted from the U6SIshRNA-transduced cells confirmed siRNA expression (data not shown). To demonstrate the effects of selected HIV-1 mutants, the culture supernatant collected on day 20, which did not show the transversion of C to A, was used to challenge fresh U6SIshRNA-transduced CEM T cells. As seen in Fig. 1D, U6SIshRNA-transduced cells showed around 99% inhibition of wild-type IIIB virus compared to cells transduced with vector alone. We have obtained a similar viral mutational breakthrough with shRNAs targeting *tat* and *vif* sites (data not shown). These mutant analyses prompted us to look for combinatorial approaches for the inhibition of HIV replication to minimize or abrogate viral resistance.

Efficient read-through occurs from the HIV-1 LTR-hsp70 fusion promoter. Since it has been previously reported (6) and confirmed in our study that HIV-1-resistant mutants arise quite readily under the selective pressure of siRNAs, we devised a two-pronged approach to inhibit HIV-1 replication. We hypothesized that we may be able to coexpress an anti-HIV-1 shRNA along with an anti-HIV-1 protein from a single Tat-inducible promoter to circumvent the resistance problem. Since the approach involved expressing a protein along with an shRNA, we used our earlier reported HIV-1-inducible LTR-hsp70 fusion promoter (36). In the previous study we reported that we could detect transcripts that originate from both the HIV-1 LTR and the hsp70 TATA box component in an HIV-1 Tat-inducible manner. However, only transcripts that originated from the hsp70 TATA box were processed to mature siRNA-sized products. This system should give rise to two mutually exclusive transcripts from each component of the fusion promoter: one that terminates at the mpoly(A) site and gets processed to functional siRNA molecules and another that is a read-through of the weak mpoly(A) signal generating a translatable message. As a first test of the hypothesis, we inserted an EGFP coding sequence coupled to a strong SV40 poly(A) signal downstream of the HIV-1 LTR-hsp70 fusion

promoter anti-*rev* shRNA-mpoly(A) unit. A strong eukaryotic translation initiation site (CCACC) was placed immediately upstream of the initiation codon of EGFP (Fig. 2A) and downstream of the shRNA-mpoly(A) sequence. HEK 293 cells were cotransfected with LTRsh-GFPpA with and without wild-type proviral DNA pNL4-3 to provide Tat *in trans*. At 72 h post-transfection, the cells were analyzed by fluorescent microscopic analysis. As seen in Fig. 2B, strong Tat-inducible expression of EGFP took place only in the presence of HIV-1. As we previously reported, transcription complexes at both the HIV-1 LTR and hsp70 basal promoters are activated by HIV-1 Tat (36). To determine whether or not the shRNA was expressed and processed into siRNA, total RNA was extracted from the cells and subjected to Northern blotting and hybridization analyses. Expression and processing of the shRNA into siRNA took place only when Tat was coexpressed with this construct (Fig. 2C). U6 RNA was probed as a loading control.

Cloning and coexpression of huRevM10 and anti-*rev* siRNA from the fusion promoter. Given that we could demonstrate coupled HIV-1 Tat-inducible expression of the anti-*rev* shRNA and EGFP, we replaced the EGFP segment with a humanized variant of the transdominant mutant RevM10 (huRevM10) (Fig. 3A). The amino acid sequence of huRevM10 is the same as that of RevM10, but the nucleic acid sequence is changed to maximize codon usage in human cells (28). These sequence changes are within the anti-*rev* shRNA target sequence and are sufficient to prevent the anti-*rev* shRNA from inactivating the M10 transcripts.

The entire cassette was inserted in the pHIV-7 lentiviral vector in the forward orientation such that transcripts reading through the minimal poly(A) would go on to transcribe huRevM10 and terminate at the lentiviral poly(A) in the 3' LTR. To test if both classes of transcripts are made in an inducible manner, HEK 293 cells were cotransfected with pNL4-3 and the HIV-7-LTRsh-RevM10 construct. Total RNA was extracted from these cells for Northern blot analysis of the shRNAs and siRNAs (sh/siRNAs) and huRevM10. Probing for the sh/siRNAs once again revealed HIV-1 Tat-inducible expression (Fig. 3B). To check for huRevM10 expression, 25 μ g of total cellular RNA was subjected to Northern gel analysis using an huRevM10-specific probe. The results show strong upregulation of the huRevM10 transcript levels in cells cotransfected with HIV-1 pNL4-3 (Fig. 3C).

Transcripts from both the LTR and hsp70 promoters were observed, appearing as an inducible doublet in cells cotransfected with pNL4-3. These data suggest that some fraction of transcripts originates via read-through of the minimal poly(A) sequence to express the downstream RevM10 gene. We do, however, see approximately a twofold higher proportion of transcripts originating from the HIV-1 LTR compared to transcripts originating from the minimal hsp70 promoter that read through the minimal poly(A) sequence.

Inhibition of HIV-1 gene expression in transient transfection assays. To test this construct in a transient setting and also to determine its potency against HIV-1, HEK 293 cells were cotransfected with pNL4-3 and the HIV-7-LTRsh-RevM10 construct. Our earlier reported LTRhsp-shRNA construct, which has the exact shRNA-mpoly(A) cassette as the HIV-7-LTRsh-RevM10 construct but without the RevM10 component, was included as a control. No p24 was detected in cells cotransfected with HIV-7-LTRsh-RevM10 (Fig. 4A), suggest-

FIG. 1. (A) Schematic representation of U6SIIshRNA cloned in the lentiviral vector pHIV-7. S, sense strand of the shRNA; L, loop; AS, antisense strand of the shRNA; WPRE, woodchuck hepatitis virus posttranscription regulation element; FLAP, sequence consisting of central polypurine tract and the central termination signal which facilitates the nuclear import of lentiviral vector. (B) Sequence analysis of anti-*rev* shRNA target sequence on day 40 following challenge of the U6SIIshRNA-expressing cells with HIV-1 IIIB. The wild-type sequence contains a cytosine at the position indicated by the arrow. This is changed to an adenosine in approximately 30% of viral RNAs compared to cells transduced with vector alone. The culture supernatant from this experiment was used to rechallenge fresh CEM T cells transduced with the U6SIIshRNA construct (U6SIIshsup to CEMSII). On day 15 viral RNA was extracted, amplified by RT-PCR, and sequenced. The C-to-A transversion represents over 80% of the viral population in the second challenge. (C) Virus from the day 40 culture was used to infect fresh cultures of vector backbone or U6SIIshRNA-transduced CEM cells. On day 15 postinfection, p24 ELISAs were performed on the culture supernatants. (D) To verify that the U6SIIshRNA-transduced cells could provide protection against wild-type virus, culture supernatant collected on day 20 of the first infection which did not show the C-to-A transversion was used to challenge fresh U6SIIshRNA-transduced CEM T cells or vector backbone cells, and on day 15 p24 ELISAs were performed.

FIG. 2. (A) Schematic representation of the LTRsh-GFPpA expression cassette cloned in pCR2.1 vector (Invitrogen). TS, transcription start; S, sense strand; AS, antisense strand; L, loop. A preferred eukaryotic translation initiation start site CCACC was placed immediately upstream of the EGFP start codon and downstream of the shRNA-mpoly(A) sequence. (B) Fluorescent microscopic analysis shows EGFP expression only in cells cotransfected with proviral DNA pNL4-3. (C) Northern blot analysis of total RNA extracted from the same cells shows efficient expression and processing of shRNA in cells transfected with LTRsh-GFPpA. The siRNA-sized products are indicated; the upper and lower arrows point to partially processed shRNA and siRNA, respectively, and as described previously (18). U6 was probed as a loading control.

ing a complete shutdown of viral replication. The level of inhibition by the HIV-7-LTRsh-RevM10 construct was higher than that observed with LTRhsp-shRNA, which by itself still showed approximately 90% inhibition of p24 levels in culture supernatant. Cells cotransfected with LTRsh-GFPpA also showed inhibition comparable to the nonfusion shRNA constructs, suggesting that cloning of an open reading frame downstream of the shRNA expression cassette does not interfere with shRNA expression and processing. Primer extension analyses using a primer flanking the huRevM10 start codon showed transcripts originating from both the promoters (Fig. 4B).

Inhibition of HIV-1 gene expression in CEM T cells stably transfected with HIV-7-LTRsh-RevM10 constructs. CEM T cells were stably transduced with the HIV-7-LTRsh-RevM10 construct (Fig. 5A). The transduced cells were challenged with HIV-1 IIIB at an MOI of 0.01. At different time points, culture supernatants were collected, and cell counts were determined by trypan blue staining. The ratio of live to total cells was determined and expressed as a survival plot. The culture supernatants were analyzed for p24 levels and expressed as picograms of p24 per cell. As seen in Fig. 5B, the cell survival ratio was greatest for HIV-7-LTRsh-RevM10. The p24 ELISAs showed an approximately 90% reduction of p24 in cells expressing the HIV-7-LTRsh-RevM10 along the entire course of the experiment (Fig. 5C). The survival plot showed a consistently higher survival ratio for cells transduced with HIV-7-LTRsh-

RevM10 than the parental HIV-7 vector control. Since the huRevM10 might mask the emergence of viral mutants in the shRNA target sequence, we collected viral RNA from cell supernatants every 10 days and carried out RT-PCR amplification and DNA sequencing analyses. Despite the fact that some virus was being produced (enough for RT-PCR amplification), DNA sequence analyses of viral RNA extracted up to day 70 did not show emergence of mutations at or near the siRNA target site (Fig. 6A). To determine the level of protection by the RevM10 component alone, the SII C-to-A mutant virus was used to challenge LTRhsp-shRNA or HIV-7-LTRsh-RevM10. At designated time points, culture supernatants were collected for p24 ELISA. As seen in Fig. 6B, no inhibition of the mutant viral replication is observed with CEM T cells transduced with LTRhsp-shRNA compared to the cells transduced with vector alone, while HIV-7-LTRsh-RevM10-transduced cells still show an approximately 80% inhibition, which is slightly lower (approximately 10%) than inhibition observed with cells challenged with wild-type IIIB.

DISCUSSION

RNAi is a potent inhibitor of targeted gene expression in a wide variety of organisms (12, 19, 34, 38). The triggers for RNAi are siRNAs that are processed from long double-stranded or hairpin RNA precursors and become part of a

FIG. 3. (A) Schematic representation of the HIV-7-LTRsh-RevM10. LTRhsp-shRNA ligated to huRevM10 is inserted directly upstream of the CMV promoter of EGFP in the pHIV-7-GFP lentiviral vector in the forward orientation (as shown by the arrow) such that transcripts reading through the minimal polyadenylation signal sequence would go on to express the RevM10 open reading frame and terminate at the lentiviral poly(A) (pA) in the 3' LTR. TS, transcription start; S, sense strand; AS, antisense strand; L, loop. (B) Northern blot analysis of total RNA extracted from cells cotransfected with HIV-7-LTRsh-RevM10 in the presence or absence of pNL4-3 shows efficient expression and processing of shRNA in the presence of pNL4-3. U6 was probed as a loading control. (C) Northern blot analysis of the total RNA extracted from the same cells and probed with an huRevM10-specific probe shows inducible expression of huRevM10 transcript. A doublet is seen, which would correspond to transcripts originating from both the LTR and the hsp70 components of the fusion promoter. GAPDH was probed as a loading control. CC, cell control.

ribonucleoprotein complex (23, 33). Most expression systems produce shRNAs from Pol III promoters, which provide discrete 5' and 3' termini and can be further processed into functional siRNAs. These transcripts mimic microRNA precursors in structure and are most probably cytoplasmically exported by the transport factor exportin 5 (42). Previous studies have demonstrated efficient inhibition of HIV-1 using Pol III-expressed sh/siRNAs (22). But as reported earlier (6, 37) and confirmed in our studies, HIV-1 viral escape mutants can arise under the selective pressure of a single shRNA. Since our aim was to inhibit HIV-1 using a combinatorial approach, we devised a strategy to coexpress an anti-HIV-1 siRNA along with an anti-HIV-1 protein from a Pol II promoter. There are several potential advantages to utilizing Pol II-expressed shRNAs, including tissue-specific promoters and inducible transcription. We previously reported an HIV-1-inducible shRNA system wherein the HIV-1 LTR including and up to the transactivation response element is fused to a

Drosophila minimal hsp70 promoter (36). In that study, shRNA expression occurs only in cells infected with HIV-1. The fusion promoter exploits the significant similarities of both the promoter elements, namely, that both the promoters are regulated by promoter-proximal pausing and that basal transcription in both of these promoters is arrested by the negative transcription elongation factor (N-TEF) which includes the negative elongation factor (NELF) and DRB (5,6-dichlorobenzimidazole riboside) sensitivity-inducing factor (13, 40). In both of these promoters, the positive transcription elongation factor P-TEFb, which is a heterodimer of Cdk-9 and cyclin T1, is recruited to the promoter to release the elongation block. P-TEFb is known to phosphorylate both the NELF and the *spt5* subunit of DRB sensitivity-inducing factor, causing the dissociation of NELF and phosphorylation of the C-terminal domain of RNA Pol II, thereby releasing the elongation block (13).

Some of the restrictions for expression of a functional

FIG. 4. (A) Inhibition of HIV gene expression by HIV-7-LTRsh-RevM10. 293 cells were cotransfected with pNL4-3 and LTRsh-GFPpA, LTRhsp-shRNA, or HIV-7-LTRsh-RevM10. The p24 levels on day 3 of pNL4-3 singly transfected cells was considered 100%, and the rest of the values were expressed relative to that value. Significant inhibition of HIV-1 gene expression is observed with the LTRhsp-shRNA and LTRsh-GFPpA constructs. Total inhibition of HIV-1 gene expression is observed with HIV-7-LTRsh-RevM10. (B) Primer extension analysis of total RNA. A primer flanking the huRevM10 transcription start site was used for primer extension analysis to determine the source of transcripts that read through the minimal poly(A) sequence. Total RNA extracted from cells cotransfected with HIV-7-LTRsh-RevM10 and pNL4-3 compared to mock-transfected cells shows transcription originating from both the HIV-1 LTR and the hsp70 components of the fusion promoter. The hsp70 TATA element is immediately upstream of the transcription start site of the hsp70-derived transcript.

shRNA from a Pol II promoter include use of a minimal poly(A) signal (41). It stands to reason that reducing the length of a poly(A) signal sequence would make it a weaker signal for polyadenylation. In the present study we exploited the possi-

bility that read-through of the shRNA and mpoly(A) sequences could be used to coexpress a downstream open reading frame. As a first test for this possibility, we inserted the EGFP gene with a full-length SV40 poly(A) sequence down-

FIG. 5. (A) Inhibition of HIV-1 replication. (A) The lentiviral vector, pHIV-7-GFP, contains a hybrid 5' LTR in which the U3 region is replaced with the CMV promoter, the packaging signal (Ψ), the RRE sequence, the EGFP gene driven by the CMV promoter, and the 3' LTR in which the *cis* regulatory sequences are completely removed from the U3 region. Thus, upon integration transcription can only initiate at the internal promoters. Arrows indicate the orientation of the individual cassettes cloned in pHIV-7-GFP. The solid box between RRE and CMV-EGFP represents the FLAP sequence of HIV-1, which consists of the central polypurine tract and the central termination signal that facilitates nuclear import of the reverse transcribed vector. (B and C) HIV-1 challenge assay. CEM cells transduced with HIV-7-LTRsh-RevM10 were challenged with HIV-1 IIIB at an MOI of 0.01. In panel B, cells were stained with trypan blue at the indicated days, and viable cell counts are plotted as live/total cell ratios. In panel C, at the designated time points culture supernatants were collected for p24 assays and cells were stained with trypan blue and counted as described in the experimental protocols to give picograms of p24/viable cell count.

A

B

FIG. 6. (A) DNA sequence analysis of HIV-1 *rev* gene targeted by the shRNA from cells transduced with the anti-*rev* shRNA/huM10 dual expression construct. No mutational alterations in the region of the shRNA target can be detected. The sequence of the same region from the HIV-7 vector-transduced, HIV-1-challenged cells is also shown. In the latter case there is no selective pressure for mutation accumulation. (B) LTRhsp-shRNA- and HIV-7- LTRsh-RevM10-transduced CEM T cells were challenged with the *rev* mutant depicted in Fig. 1. On day 15 culture supernatants were collected for a p24 assay. Vec, vector.

stream of the mpoly(A) in two different shRNA expression cassettes. Efficient expression of EGFP as well as the shRNA was observed only in presence of wild-type HIV-1 pNL4-3. The observed inhibition of p24 levels was comparable to what was

previously reported with our LTRhsp-shRNA construct (36). These results suggested that cloning of EGFP downstream of the mpoly(A) does not affect the processing of the shRNA or its inhibitory potential. The shRNA-reporter construct-induc-

ible coexpression should prove useful in cell culture experiments for identifying HIV-1-infected cells, which in turn would provide a more accurate analysis of the effectiveness of the siRNA-mediated inhibition of HIV-1 replication.

In order to test whether a combination of anti-HIV shRNA and anti-HIV transdominant protein could be coexpressed in the HIV-1 Tat-inducible system, we inserted the huRevM10 gene downstream of the shRNA-mpoly(A) element. The rationale behind this was to provide a combinatorial therapy using two different antiviral genes. The anti-*rev* shRNA has already been demonstrated as an effective anti-HIV agent, but clearly HIV-1 viral escape mutants can arise under the selective pressure of a single shRNA (6).

By introducing the huRevM10 construct into the same vector, the replicative ability of the virus should be severely impeded, thereby reducing the probability of escape mutants. It has already been demonstrated that prolonged infection of HIV-1 in cells expressing either an shRNA or RevM10 alone can result in the emergence of viral escape mutants (6, 17, 37). Another potential advantage of our inducible system in a real gene therapy setting is that RevM10 would only be expressed in HIV-1-infected cells, thereby reducing the potential for immunogenicity of this HIV protein in immune cells.

When HEK 293 cells were cotransfected with HIV-1 pNL4-3 and HIV-7-LTRsh-RevM10, total inhibition of HIV-1 p24 antigen expression was observed. We have previously demonstrated that even anti-*rev* si/shRNAs alone are potent inhibitors of viral replication in a transient setting as well as in a long-term gene therapy setting in both CEM T cells and CD34 stem cells (20, 36). The transdominant effect of RevM10 only adds to the inhibitory effect by blocking expression of late viral RNAs harboring the Rev binding element. Cells transduced with HIV-7-LTRsh-RevM10 in a lentiviral vector showed approximately 90% inhibition of HIV-1 p24 expression and a consistently higher cell survival ratio, thus establishing the potential use of this expression system in a gene therapy setting. Moreover, by suppressing viral replication via two different mechanisms (RNAi and transdominant M10), our combinatorial approach further restricted the emergence of viral mutants during the entire course of our experiment. When cells transduced with HIV-7-LTRsh-RevM10 in a lentiviral vector were challenged with the SII mutant virus, we still observed an approximately 80% inhibition of the virus, suggesting that in the event of mutants arising against any one component, the other component would still inhibit viral replication. Beyond the applications to HIV-1, the dual expression approach should have applications in other venues. There are clear advantages to having both the shRNA and open reading frame coexpressed from the same promoter, aside from the fact that both sequences can be inserted into vectors with limited cloning capacities. For instance, confirmation that a given phenotype was truly elicited by the si/shRNA in question could be made by coexpressing an shRNA with an RNAi-resistant, codon-modified version of the target gene that restores the original cellular phenotype. This technique could also be applied to deliver corrective genes and simultaneously knock out deleterious gene expression using shRNA expression systems in the appropriate disease models, and the same tissue-specific or inducible promoter can be used to regulate the expression of the shRNA and protein to only the cells or tissues of interest.

Moreover, this technique could be adapted to any expression system where one effector arm is an RNA (e.g., siRNA, aptamer, or ribozyme) and the other is a protein.

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