A U1i RNA that Enhances HIV-1 RNA Splicing with an Elongated Recognition Domain Is an Optimal Candidate for Combination HIV-1 Gene Therapy

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U1 interference (U1i) RNAs can be designed to correct splicing defects and target pathogenic RNA, such as HIV-1 RNA. In this study, we show that U1i RNAs that enhance HIV-1 RNA splicing are more effective at inhibiting HIV-1 production compared to top U1i RNAs that inhibit polyadenylation of HIV-1 RNA. A U1i RNA was also identified targeting a site upstream of the first splice acceptor site in the Gag coding region that was effective at inhibiting HIV-1 production. U1-T6, which enhanced HIV-1 RNA splicing, was superior to an antiviral short hairpin RNA (shRNA) currently in clinical trials. To increase specificity, the recognition domain of U1-T6 was elongated by 3–6 nt. The elongated molecules inhibited HIV-1 production from different HIV-1 strains, including one with a mismatch in the target site. These results suggest that lengthening the recognition domain can enhance the specificity of U1i RNAs for their intended target sites while at the same time allowing them to tolerate single mismatch mutations. Overall, our results demonstrate that U1-T6 with an elongated recognition domain inhibits HIV-1 production and has both the efficacy and specificity to be a promising candidate for HIV-1 gene therapy.

INTRODUCTION

Current HIV-1 treatment consists of a combination of antiretroviral therapies (cART), which can successfully control HIV-1 replication and prevent the onset of AIDS.1 While cART has drastically improved the lifespan of individuals infected with HIV-1, it does have limitations, including drug-related toxicity, patient compliance issues, high costs, and emergence of viral resistance.2 Moreover, the persistence of viral latent reservoirs during cART leads to rapid rebound of viremia following treatment cessation, requiring cART to be a lifelong therapy.3 Due to these well-known limitations associated with current HIV-1 therapy, a cure for HIV-1 infection remains a highly desirable goal to improve the lives of HIV-1-infected individuals.

So far, the only procedure that has resulted in a cure for HIV-1 infection is an allogeneic hematopoietic stem cell (HSC) transplant from an HIV-1-resistant donor. In one case, the patient has been off antiretroviral therapy with no viral rebound for 12 years,4,5 and in a second recently reported case, the patient has been off therapy for 18 months with no viral rebound.6,7 Due to a limited number of individuals with known resistance to HIV-1 and risks associated with HSC transplant between individuals, a substantial amount of research has focused on using an autologous transplantation of ex vivo genetically modified HSCs to make these remarkable cases of an HIV-1 cure available to all infected individuals. In this approach, patient-derived HSCs are purified, expanded, and transduced with antiviral RNAs such as short hairpin RNAs (shRNAs),8 ribozymes,9 and decoy and aptamer RNAs,10 designed to target and reduce HIV-1 replication. These cells are then re-infused, providing patients with a persistent source of HIV-1-resistant lymphoid and myeloid cell lineages. However, viral escape in such an approach remains a significant concern.11 As with cART, gene therapy will require a combination of antiviral genes to prevent the development of resistant viruses. Although several clinical trials (reviewed in Scarborough and Gatignol11) have begun, there remains a need for the identification and characterization of novel and potent antiviral RNAs.

The U1 small nuclear RNA (U1 snRNA), in complex with seven Smith (Sm) proteins and three U1-specific proteins (U1-70K, U1-A, and U1-C), is a fundamental component of the spliceosome, a ribonucleoprotein (RNP) complex that catalyzes precursor mRNA splicing.12 During the early steps of spliceosome assembly, 5′ splice donor sites (5′ss) of pre-mRNAs are recognized by the U1 snRNA through RNA-RNA interactions with the 5′ recognition domain of the U1 snRNA (Figure 1A). U1 small nuclear RNP (snRNP) binding, along with the recognition of the upstream 3′ splice acceptor sites (3′ss) polypyrimidine tract (PPyT) by the U2AF heterodimeric cellular splicing factor and the branch point sequence by branch point binding protein (SF1/mBBP), allows for recruitment of the...
U2 snRNP and proper formation of the spliceosome’s catalytic core. Spliceosomal assembly across exons leads to splicing by a process termed “exon definition.” The U1 snRNP has also been implicated in repressing 3′ end polyadenylation of pre-mRNAs via interactions with cis elements located upstream or downstream of polyadenylation sites (PASs). Inhibition of 3′ end processing is mediated by interactions between U1-specific U1-70K protein and the poly(A) polymerase (PAP). Transcripts that lack a poly(A)
Table 1. Summary of U1i RNA Target Sites in HIV-1

| U1i RNA Name | Primer (5′ → 3′) | Target Sequence (5′ → 3′) | Location | Reading Frame |
|--------------|-----------------|------------------------|----------|--------------|
| U1-Gag1      | GCCCCAAGATCTCACTAGTCCCTGCAGG | GCAGGAACTA | 1498 | Gag |
| U1-Gag2      | GCCCCAAGATCTCACTAGTCCTGAGGGAGG | CAGGAACACTA | 1499 | Gag |
| U1-Gag3      | GCCCCAAGATCTCACTAGTGTTAGGAGGAGG | ACTCTAGTA | 1504 | Gag |
| U1-Gag4      | GCCCCAAGATCTCACTAGGTTAGGAGGAGG | ACTAGTACC | 1507 | Gag |
| U1-D2        | GCCCCAAGATCTCAACCTCACCCTGAGGGAGG | AGGTGAG | 4961 | Gag (D2) |
| U1-D3        | GCCCCAAGATCTCAACTCTACTGAGGGAGG | AGGTAAG | 5262 | Vif (D3) |
| U1-T3        | GCCCCAAGATCTCTAGTTAGGGCTGTGAGGGAGG | GGAAGCATCCA | 5861 | Tat/Vpr |
| U1-T4        | GCCCCAAGATCTCACTATAGTTAGGGCTGTGAGGGAGG | GTCAAGCTAAA | 5876 | Tat |
| U1-T6        | GCCCCAAGATCTCACTATTAGGGCTGTGAGGGAGG | CTGCTTGTAA | 5887 | Tat |
| U1-Rev       | GCCCCAAGATCTCACTAGTGAAGTTAGGGAGGAGG | CTTCAAGCTAC | 8509 | Rev exon 2 |
| U1-Env       | GCCCCAAGATCTCACTATTAGGCTGAAGTTAGGGAGGAGG | TTCAGCTAC | 8776 | Env |
| U1-Nef       | GCCCCAAGATCTCAACTCAGGGATGCGAGGAGGAGG | CATCCGGAGT | 9381 | LTR/Nef |

*Position of targeted sequence is relative to HIV-1 strain NL4-3.

Tail are inherently unstable and are rapidly degraded by the host cell machinery.17

U1 interference (U1i) is a technique used to inhibit the expression of a targeted gene by exploiting the properties of the U1 snRNP to inhibit 3′ end polyadenylation when targeting 3′ terminal exons or by enhancing splicing when bound to a 5′ss or downstream of a 3′ss by the process of exon definition. Inhibition is achieved by modifying the 5′ recognition domain of U1 snRNAs to contain sequences complementary to regions in the terminal exon or downstream of a 3′ss of a targeted transcript (Figure 1B). These modified U1 snRNAs are often referred to as U1i RNAs,18 and some studies have shown that they have a synergistic inhibitory effect on mRNA expression when combined with other U1i RNAs or shRNAs.19,20 Modified U1 snRNAs have also been designed to correct aberrant splicing in several genetic diseases.21,22

To date, there have been three independent studies utilizing U1i RNAs to inhibit HIV-1 replication. Two of these studies designed U1i RNAs targeting highly conserved sequences in the 3′ end of HIV-1 RNA and demonstrated that U1i RNAs that inhibit polyadenylation can inhibit HIV-1 production in cell culture.23,24 The other study demonstrated that U1i RNAs designed to enhance HIV-1 splicing, by targeting 5′ss or sites downstream of 3′ss, could also inhibit HIV-1 production in cell culture.25 However, these U1i RNAs have shown mixed effects against HIV-1 replication in T lymphocytic cell lines, with one study demonstrating no efficacy against HIV-1 replication.24,25 While all three of these studies identified anti-HIV-1 U1i RNAs, there has yet to be a study comparing the efficacy of U1i RNAs with different mechanisms of action.

In this study, we constructed new U1i RNAs targeting the Gag coding sequence along with the top candidates from previous studies and compared their inhibitory effects on the production of HIV-1. U1i RNAs that enhance splicing were more potent compared to those that inhibit polyadenylation, with the most potent molecule targeting a site downstream of the third splice acceptor site in HIV-1 RNA, named U1-T6. Unexpectedly, some U1i RNAs designed to inhibit polyadenylation also enhanced HIV-1 RNA splicing, while a U1i RNA targeting the Gag region did not enhance splicing. In Sup-T1 T lymphocytes, U1-T6 transduced cells were the most effective at restricting HIV-1 replication and were able to restrict replication for a longer time compared to an shRNA currently in clinical trials for HIV-1 gene therapy.26 Increasing the recognition domain of U1-T6 by 3–6 nt did not affect its antiviral potency, enhanced its calculated target site specificity, and allowed it to remain potent against an HIV-1 strain with a single mismatch in the target site. Overall, our results suggest that U1-T6 with an elongated recognition domain is a competitive candidate for use in combination gene therapy for HIV-1 and that increasing the recognition domains of U1i RNAs can be advantageous for their development as therapeutics or as biotechnology tools.

### RESULTS

**U1i RNAs Targeting the Gag Coding Sequence of HIV-1 RNA Are Effective Inhibitors of HIV-1 Production**

For antisense-based molecules targeting HIV-1 RNA, an important consideration for their development as therapies is that they target highly conserved sequences. In a previous study, we identified a conserved target site in the Gag coding sequence of HIV-1 RNA that was accessible to inhibition by a ribozyme and an shRNA.27 To determine whether the target sequence is also accessible to inhibition by U1i RNAs, four U1i RNAs (U1-Gag1, 2, 3, and 4) were designed, targeting overlapping regions within the Gag target site (Table 1; Figure 2A). The effects of U1i RNAs on HIV-1 production were evaluated by co-transfection of HEK293T cells with HIV-1 molecular clone pNL4-3 and increasing concentrations of U1i RNA constructs. HIV-1 production was compared by measuring the activity of the HIV-1 reverse transcriptase (RT) enzyme in culture supernatants, and data were expressed as a percentage of RT activity in cells co-transfected with the wild-type (WT) U1 snRNA-expressing construct. In parallel,
cells were co-transfected with pNL4-3 and the shRNA we generated in our previous study, shGag. Data were expressed as a percentage of RT activity in cells co-transfected with the empty shRNA expression plasmid. Out of the four U1i RNAs targeting Gag, U1-Gag1 and U1-Gag2 dose dependently inhibited HIV-1 production, with U1-Gag2 providing the most potent inhibition, close to the potency observed for shGag (Figure 2B). To determine whether the U1-expressing plasmid (UBC) itself had effects on HIV-1 production, we generated a new plasmid where the U1 WT sequence was replaced by a short random sequence (UBC-empty). In cells cotransfected with pNL4-3 and the U1-WT or UBC-empty plasmids at 100 ng, viral production was similar (Figure S1), suggesting that the U1-WT molecule does not by itself affect HIV-1 production.

U1i RNAs that Enhance HIV-1 RNA Splicing Are More Potent Inhibitors When Compared to U1i RNAs that Inhibit Polyadenylation

To directly compare the effects of U1i RNAs targeting HIV-1 RNA, we used the top three U1i RNAs designed to inhibit polyadenylation, identified in the initial U1i RNA screen, and the top five candidates designed to enhance splicing of HIV-1 RNA. U1i RNAs that inhibit polyadenylation all target the 3' proximal regions of HIV-1, while those that enhance splicing target 5'ss D2 or D3 and sequences downstream of the first, second, and third 3'ss, A1, A2 and A3. The target sequences for the U1i RNAs are provided in Table 1, and their positions within the HIV-1 RNA open reading frames are illustrated in Figure 3A. All U1i RNAs inhibited HIV-1 production in a dose-dependent manner (Figure 3B). Among them, U1-Gag2 and U1i RNAs designed to enhance splicing (U1-D2, T3, T4, T6) were found to be the most potent candidates, providing a 50% inhibition of viral production at around 5 ng or less of input DNA, compared to around 25 ng for U1i RNAs that inhibit polyadenylation (U1-Env, Ref, Nef). Of the U1i RNAs that inhibited polyadenylation, only U1-Env was able to provide a nearly complete inhibition of viral production at higher doses, similar to U1i RNA molecules that enhance viral RNA splicing and U1-Gag2. These results suggest that U1i RNAs acting through mechanisms other than polyadenylation inhibition are more potent at inhibiting HIV-1 production.

Using the HIV Los Alamos database and previously developed methods, we estimated the conservation at the nucleotide level of sites targeted by U1i RNAs among all complete HIV-1 sequences available in the database (3,666 at the time of analysis). Sites targeted by U1-D2, U1-D3, and U1-Gag2 had the highest conservation among the HIV-1 strains with more than 90% conservation at each nucleotide (Figure 3C). Sites targeted by U1-Env, U1-Rev, U1-Nef, and U1-T6 had an intermediate level of conservation with most nucleotides conserved in greater than 80% of the strains and sites targeted by U1-T3 and U1-T4 had the lowest conservation, with two nucleotides conserved in less than 80% of the strains (Figure 3C).

A significant concern for the development of antiviral RNAs for use in gene therapy is their potential to cause cellular toxicity, as this can alter the interpretation of experimental results and limit their potential for use in clinical settings. To evaluate whether the reduction of HIV-1 production seen when expressing U1i RNAs is due to toxicity in HEK293T cells, we assessed the ability of U1i RNAs to affect cell viability. HEK293T cells were transfected with U1i RNAs at 1,000 ng/mL cultured supernatant, a dose several fold higher than their maximal inhibitory concentrations. Cell viability was estimated using the WST-1 colorimetric assay, and data were expressed as relative WST-1 metabolism compared to cells transfected with the U1-WT construct. While treatment of cells with H2O2 resulted in significant cell death, there were no major differences between U1i RNA-transfected cells and U1-WT-transfected cells (Figure S2). Based on the lack of cellular toxicity, conservation of their target sites and their ability to inhibit HIV-1 production, U1-Gag2, D2, T6, Rev, Env, and Nef were selected for further evaluation.

HIV-1 Suppression by All U1i RNAs Is Dependent on the Proper Function of the SL1 Domain

The endogenous U1 snRNA molecule exerts its function in the form of a RNP complex that consists of three specific U1 proteins, U1-A, U1-70K, and U1-C, and seven Sm proteins. To determine whether the inhibition of HIV-1 production by the U1i RNA molecules was dependent on proper assembly of the U1 snRNP, we mutated the stem loop 1 (SL1) domain of all the U1i RNAs in a manner that is expected to result in a loss of U1-70K binding and the SL2 domain to result in the loss of U1-A binding. The mutated sequences are illustrated in Figure 1A. While U1-70K binding, and to a lesser extent U1-A binding, is required for inhibition by U1i RNAs that inhibit polyadenylation, the U1i RNAs that enhance HIV-1 RNA splicing are more potent at inhibiting HIV-1 production. A two-way ANOVA with a Bonferroni post-test was used to compare replicate means to the means of U1-WT or empty shRNA expression-plasmid-transfected cells. The level of significance is indicated above each mean value that was significantly different from its control (*p < 0.05, **p < 0.01, ***p < 0.001).
snRNP domain requirements of U1i RNAs that enhance splicing have yet to be determined.\textsuperscript{16,23}

The effects of the SL1 and SL2 mutants on HIV-1 production were compared with the non-mutated versions of the selected U1i RNAs at different doses (Figure 4). Little to no inhibition was observed for the SL1 mutants at all doses evaluated. These results suggest that the inhibition of HIV-1 production by all U1i RNAs evaluated was dependent on the formation of a U1 snRNP complex and not the result of antisense RNA effects alone. At higher doses, SL1 mutants, U1-D2 and U1-T6, had some effects on HIV-1 production (Figures 4A and 4B). This could be the result of antisense effects or may be related to competitive inhibition of 3'\textsuperscript{ss} or 5'\textsuperscript{ss} by mutated U1i RNAs, impeding the normal binding and subsequent splicing by the spliceosome complex. Mutation of the SL2 domain had little to no effect on U1-D2 and U1-T6, which were designed to enhance splicing (Figures 4A and 4B), suggesting that binding of the U1 SL2 loop to the U1-A protein is dispensable for their effects. Similar results were obtained for U1-Env and U1-Gag2 (Figures 4C and 4D). In contrast, SL2 mutations abolished the inhibitory effects of U1-Rev and U1-Nef (Figures 4E and 4F).

\textbf{U1i RNA Expression Alters HIV-1 RNA Accumulation}

We next compared effects of the selected U1i RNAs on HIV-1 RNA accumulation by northern blot following co-transfection with pNL4-3 in HEK293T cells (Figure 5A). Consistent with previous results,\textsuperscript{25} co-transfection with U1-D2 and U1-T6 resulted in a nearly complete reduction of full-length (FL) HIV-1 RNA and an increase in incompletely spliced (IS) or both IS and completely spliced (CS) RNA species. In contrast, U1-Gag2, U1-Rev, and U1-Nef expression resulted in a decrease of all RNA species, suggesting that these molecules do not enhance HIV-1 RNA splicing. Unexpectedly, U1-Env expression resulted in an increase in CS RNA, implying that at least part of its effects on HIV-1 production can be attributed to its ability to enhance HIV-1 RNA splicing. This effect may also explain why, of the molecules designed to inhibit polyadenylation, it was the only one that completely inhibited HIV-1 production (Figure 3B) and the only one that was not affected by the SL2 mutation (Figure 4C versus Figures 4E and 4F).

To further explore the effects of U1i RNAs on HIV-1 RNA expression, we constructed cDNA libraries from the RNA extracted from co-transfected HEK293T cells and used previously described primer sets\textsuperscript{25,31} to amplify CS and IS cDNAs. The positions of these primers and major exons used for the generation of HIV-1 RNA splice variants are illustrated in Figure 5B. The products of the amplification were run on an agarose gel and visualized with UV light (Figure 5C). Consistent with previous results,\textsuperscript{25} U1-D2, targeting the 5'\textsuperscript{ss} D2, increased inclusion of exon 2 or exon 2E. This is shown by the increase in CS mRNA species 1.2.5.7 (\textit{nef}3) and IS 1.2E (\textit{vif}2) and 1.2.5E (\textit{env}5) and a decrease in other viral mRNA species. Also consistent with the previous study,\textsuperscript{25} U1-T6, targeting the 5'\textsuperscript{ss} D2, increased inclusion of exon 2 or exon 2E. This is shown by the increase in CS mRNA species 1.2.5.7 (\textit{nef}3) and IS 1.2E (\textit{vif}2) and 1.2.5E (\textit{env}5) and a decrease in other viral mRNA species. Also consistent with the previous study,\textsuperscript{25} expression of U1-T6 resulted in increased inclusion of exon 4 or exon 4E. This is demonstrated by the increase in CS species 1.4.7 (\textit{Tat}1), 1.2.4.7 (\textit{Tat}2), and 1.3.4.7 (\textit{Tat}3), as well as IS species 1.4E (\textit{Tat}5). As seen with the northern blot results, U1-Env had major effects on the splicing pattern of HIV-1 RNA. The results suggest that similar to U1-T6, U1-Env acts through increasing inclusion of exon 4 and, to a lesser extent, exon 4E. This is shown by the increased
expression of CS species 1.4.7 (Tat1) and 1.3.4.7 (Tat3) and IS species 1.4E (Tat5) (Figure 5C). Although there were no apparent effects of U1-Rev on splicing in the northern blot (Figure 5A), a small effect was detected when we looked at the IS species, where a modest increase in 1.4E was observed, suggesting that, similar to U1-Env and U1-T6, U1-Rev can lead to an increase in the inclusion of exon 4E. These results demonstrate that some U1i RNAs targeting the terminal exon of HIV-1 RNA can enhance HIV-1 RNA splicing and that some of their effects on HIV-1 production can be attributed to this mechanism. The results also demonstrate that enhanced HIV-1 RNA splicing does not contribute to the inhibition of HIV-1 production provided by U1-Gag2 and U1-Nef.

Cells Transduced with U1-T6 Demonstrate Superior Inhibition of HIV-1 Replication when Compared to Cells Transduced with an shRNA in Clinical Trials

To evaluate the potential of U1i RNAs to inhibit HIV-1 replication, we used an HIV-1-based lentiviral vector expressing an EGFP reporter gene (HIV-7)\(^{12}\) to transduce Sup-T1 cells. Following transduction, cells were sorted for GFP expression to obtain similar populations of transduced cells and eliminate high- and low-expressing cells (gating shown in Figure S3). The sorted cells were infected with a high (Figure 6A) or low (Figure 6B) amount of HIV-1 NL4-3, and viral replication was monitored by measuring HIV-1 RT activity in culture supernatants. At a high inoculum, only U1-T6-transduced cells restricted viral replication compared to control-transduced cells (Figure 6A), while at a lower inoculum both T6- and D2-transduced cells restricted viral replication compared to control cells (Figure 6B). To confirm the results with U1-T6 and compare its effects to an shRNA, a second set of cells were transduced with U1-T6, U1-WT, a nonsense shRNA (shRNA-NS), and an shRNA targeting the tat/rev region of HIV-1 RNA (shRNA-tat/rev) that has advanced to clinical trials (ClinicalTrials.gov: NCT01961063, NCT02337985, and NCT00569985). U1-T6-transduced cells again restricted viral replication compared to control-transduced cells (Figure 6C). Compared to shRNA-tat/rev-transduced cells, U1-T6-transduced cells restricted

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Figure 4. HIV-1 Suppression by All U1i RNAs Is Dependent on the Proper Assembly of the SL1 Domain and Recruitment of U1-70K whereas Only U1i RNAs that Inhibit Polyadenylation Depend on the SL2 Domain and Recruitment of U1-A

The effects of U1 snRNA SL1 and SL2 domain mutations on the suppression of HIV-1 production by U1-D2 (A), U1-T6 (B), U1-Env (C), U1-Gag2 (D), U1-Rev (E) and U1-Nef (F). HEK293T cells were co-transfected with HIV-1 pNL4-3 (100 ng) and 1–500 ng of mutated U1i RNAs. Results are expressed as a percentage of RT activity in SL1 or SL2 WT plasmid-transfected cells. Data from Figure 3 for the non-mutated U1i RNAs are shown for comparison. Data were log transformed and a nonlinear regression log(inhibitor) versus response equation with least-squares (ordinary) fit was determined using GraphPad prism. All data points are represented as the mean ± SEM of at least three independent transfections with two to three replicates (n = 6–9).
viral replication for a longer time, either because of a stronger antiviral effect or a longer delay in the development of resistant virus.

A competitive cell growth assay was used to evaluate whether the expression of U1-T6 had effects on cell proliferation. The methods used were similar to those described in the literature for comparing effects of transduced genes on cell proliferation. The sorted cells were mixed with non-transduced Sup-T1 cells to get approximately 50% GFP-positive cells and the percentage of GFP-positive cells was monitored over 50 days (Figure 6D). There was some variability between the different cultures at the first time point and the percentages of GFP-positive cells were lower in all cultures at the second time point. The variability is likely due to variations in cell counting, and the decrease may be related to effects of transduction or cell sorting on the GFP-positive cells, giving them an initial growth disadvantage compared to the non-transduced and non-sorted Sup-T1 cells. Overall, no major change in the percentage of GFP-positive cells were observed for any cultures from day 22 to day 50, suggesting that expression of U1-WT, shRNA-NS, shRNA-tat/rev, and U1-T6 do not affect cell proliferation in Sup-T1 cells.

U1-T6 Maintained Antiviral Efficacy Following Elongation of Its Recognition Domain

U1i RNAs typically have recognition domains of 9–11 nt, and increasing the length of these domains could improve the specificity of U1i RNAs for their intended target sites. Increasing the recognition domain by 6 nt did not severely compromise the inhibitory effect of a U1i RNA in one study, but severely limited the inhibitory activity in another one. Importantly, these studies demonstrated that elongated U1i RNAs are expressed at similar levels to their WT counterparts. To evaluate the impact of increasing the recognition domain of U1-T6, we increased its recognition domain by 3 or 6 nt going toward the 3′ or 5′ end of the viral genome. These length variants were named according to the direction of the increased recognition domain (toward the 5′ or 3′ end of the viral genome) and by the number of nucleotides added (5′ + 3, 5′ + 6, 3′ + 3, and 3′ + 6) and were compared to the original U1-T6 for effects on HIV-1 production (Figure 7A).

Our results show that U1-T6 maintained its ability to inhibit viral production when the recognition domain was increased toward either end of the viral genome, and by the number of nucleotides added (5′ + 3, 5′ + 6, 3′ + 3, and 3′ + 6) and were compared to the original U1-T6 for effects on HIV-1 production (Figure 7A).

These length variants were named according to the direction of the increased recognition domain (toward the 5′ or 3′ end of the viral genome) and by the number of nucleotides added (5′ + 3, 5′ + 6, 3′ + 3, and 3′ + 6) and were compared to the original U1-T6 for effects on HIV-1 production (Figure 7A).
antiviral efficacy but may depend on the site targeted and possibly the mechanism of action of the U1i RNA molecule. Nonetheless, elongated U1-T6 maintained antiviral efficacy, further demonstrating that U1i RNAs are attractive antiviral RNA molecules that merit further development.

Elongated U1-T6 Molecules Enhance Target Site Specificity and Increase Tolerance to a Single Mismatch Mutation in Their Target Site

To determine the specificity of U1-T6 and elongated U1-T6 molecules, their antiviral effects on HIV-1 production were evaluated by co-transfection of HEK293T cells with two different HIV-1 molecular clones (MJ4 and ADA) and increasing concentrations of U1-T6 constructs. MJ4 and ADA molecular clones were chosen, as they have mutations within or around the original 9-nt target site (Figures 7B and 7C). A previous study demonstrated that single base pair mismatches in positions 3–8 of the U1i RNA recognition domain that differ from GU completely abrogate the silencing activity of U1i RNAs that inhibit polyadenylation.35 However, such studies have not been conducted on U1i RNAs that enhance splicing nor on U1i RNAs with elongated recognition domains.

In agreement with previous results,35 a single base pair mismatch at position 3 of the original U1-T6 target site in HIV-1 strain MJ4 resulted in a drastic reduction of U1-T6 antiviral potency when compared to NL4-3 (Figures 7A and 7B). U1-T6 variants 3’+3 and 3’+6 with an additional two or four mismatches had no effects on viral production, demonstrating that mismatches in the elongated region can completely abrogate the effects of a U1i RNA. U1-T6 variants 5’+3 and 5’+6 with perfectly matched elongated regions were more potent inhibitors of MJ4 production compared to U1-T6, demonstrating that the deleterious effects of a single mismatch can be overcome with longer 5’ recognition domains.

U1-T6 inhibited production of HIV-1 strain ADA with similar potency compared to NL4-3 (Figures 7A and 7C). A drastic reduction in potency was observed for U1-T6 variants 3’+3 and 3’+6 with an additional two or four mismatches had no effects on viral production, demonstrating that mismatches in the elongated region can completely abrogate the effects of a U1i RNA. U1-T6 variants 5’+3 and 5’+6 with perfectly matched elongated regions were more potent inhibitors of MJ4 production compared to U1-T6, demonstrating that the deleterious effects of a single mismatch can be overcome with longer 5’ recognition domains. U1-T6 inhibited production of HIV-1 strain ADA with similar potency compared to NL4-3 (Figures 7A and 7C). A drastic reduction in potency was observed for U1-T6 variants 3’+3 and 3’+6 with two mismatches in the elongated region, further demonstrating that mismatches in the elongated regions can reduce the effectiveness of U1i RNAs (Figure 7C). In contrast, only a moderate reduction in potency was observed for U1-T6 5’+3 and 5’+6 variants. The elongated target domain of U1-T6 5’+3 and 5’+6 in ADA contained two A to G base pair mismatches, which ends up forming two GU wobble base pairs with the U1i RNA. Therefore, the
maintenance of antiviral potency when co-expressing U1-T6 +3 or +6 with ADA can be due to the formation of two GU base pairs, which are known to contribute to RNA:RNA helix stability.35,36 Overall, our results demonstrate that increases in the recognition domain of a U1i RNA can overcome a loss of effect resulting from one mismatch in the target site and that mismatches in the elongated region of two or more that differ from GU can drastically reduce the effects.

To estimate the potential contribution of lengthening the U1-T6 recognition domain to reduce off-target effects, we used GGGenome (http://gggenome.dbcls.jp/hs_reseq70/) to search for perfect matches in the NCBI RefSeq human RNA release 70, as previously described for antisense oligonucleotide sequences.37 As expected, lengthening the recognition domain greatly decreased the number of potential matches, from 1,827 perfect matches for U1-T6 to no perfect match and 1 perfect match for U1-T6 +3, +6, +3 +3, and +6 +6. Results are expressed as a percentage of HIV-1 reverse transcriptase (RT) activity in culture supernatants relative to supernatants from cells co-transfected with pNL4-3, pMJ4, or pADA and the U1-WT plasmid. All data are represented as the mean ± SEM of at least three independent transfections with two to three replicates (n = 6–9). A two-way ANOVA with a Bonferroni post-test was used to compare replicate means to the means of U1-WT-plasmid-transfected cells. The level of significance is indicated above each mean value that was significantly different from its control (*p < 0.05, **p < 0.01, ***p < 0.001).

DISCUSSION

Similar to current antiretroviral therapy, successful anti-HIV-1 gene therapy will require a combinatorial approach to prevent the development of resistant viruses.38 While several RNA-based antiviral genes are being tested in pre-clinical and clinical settings,39–42 saturation of cellular pathways, suboptimal antiviral inhibition, and cellular toxicity remain significant concerns.43–45 Several U1i RNA molecules targeting the 3’ terminal exon of HIV-1 transcripts were shown to be potent inhibitors of HIV-1 production, including the U1-Rev, U1-Env, and U1-Nef molecules evaluated in this study.23,24 However, when transduced into a T lymphocyte cell line they were not able to inhibit HIV-1 replication.24 The authors of that study concluded that at transduction levels in which an shRNA could effectively inhibit HIV-1 replication, the expression levels of the U1i RNAs were not high enough to inhibit replication and that they would therefore
not be competitive candidates for gene therapy without further improvements.

The main goal of this study was to compare the most potent U1i RNAs to date and to determine the best molecule(s) to be used in an anti-HIV-1 gene therapy setting. Therefore, we compared new and previously characterized U1i RNAs for their ability to inhibit HIV-1 production when transfected at various doses. Based on our previous studies, we found a highly conserved target site in the Gag open reading frame that was highly accessible to inhibition mediated by an shRNA and a ribozyme. Therefore, we were also curious to see whether U1i RNAs targeting this site can inhibit HIV-1 production. We identified one U1i RNA (U1-Gag2) that inhibited HIV-1 production with potency close to that of an shRNA and a ribozyme.27,46 Therefore, we were also curious to see whether U1i RNAs targeting this site can inhibit HIV-1 production. We identified one U1i RNA (U1-Gag2) that inhibited HIV-1 production with potency close to that of an shRNA targeting the same sequence (Figure 2). The effect of U1-Gag2 was dependent on the integrity of the SL1 domain (Figure 4D), suggesting that its mechanism of action depends on proper association of the U1-70K protein with the SL1 domain.16,30,47 When we directly compared the potency of all selected U1i RNAs, we found that U1-Gag2 was more potent than those that inhibit HIV-1 RNA polyadenylation, but that U1i RNAs that enhance splicing were the most potent of all (Figure 3B).

As with U1-Gag2, the viral inhibition provided by U1i RNAs that enhance splicing, and those that inhibit polyadenylation, was dependent on the integrity of the SL1 domain (Figure 4). SL2 mutants of U1i RNAs designed to enhance splicing and U1-Gag2 had similar potency compared to non-mutated constructs (Figures 4A, 4B, and 4D), suggesting that U1-A binding to their SL2 domain is dispensable for their mechanism of action. Similarly, U1-A binding has been found to be unnecessary in modified U1 snRNAs designed to correct exon skipping.47 In contrast, SL2 mutants of U1-Rev and U1-Nef, which inhibit polyadenylation, were unable to inhibit viral production (Figures 4E and 4F). Previously, Sajic et al.23 showed that mutations to the SL2 domain of U1i RNA molecules targeting the 3' terminal exon resulted in a reduction of viral inhibition but did not completely abolish their inhibitory effects. However, their experiments were conducted with the molecular clone HxBruR7/RI, which contains deletions in the RT and integrase genes, and they compared levels of intracellular Gag expression to measure the inhibitory effects of mutated and non-mutated U1i RNAs. In their experiments, the non-mutated U1i RNAs completely inhibited intracellular Gag expression, whereas in our experiments the non-mutated U1-Nef and U1-Rev inhibited viral production by a maximum of around 50% (Figures 4E and 4F). It is possible that in our experiments the antiviral effects of the SL2 mutants were not strong enough to observe a significant effect on viral production as was observed in their study looking at Gag expression.23 While we cannot exclude that the U1i RNAs inhibiting polyadenylation can function without binding to the U1-A protein, our results suggest that U1-A binding is important for their function (Figure 4).

The most potent U1i RNAs were found to alter the expression of viral RNA isoforms. The expression of U1-D2 resulted in an increase in IS HIV-1 RNA (Figure 5A) and inclusion of exon 2 (Figure 5C), while expression of U1-T6 resulted in an increase of both IS RNA and CS viral RNA and an increase in inclusion of exon 4. In addition, U1-Env, which was previously characterized to inhibit HIV-1 polyadenylation, enhanced splicing in our study, as demonstrated by the increase in CS RNA (Figure 5A) and increase in mRNA species containing exon 4 (Figure 5C). One potential mechanism by which U1-Env enhances HIV-1 splicing can be by increasing the recognition of splice acceptor site A7 via recruiting U2AF to the viral transcript. During HIV-1 replication, exclusion of the intron between 5' splice donor D4 and 3' splice acceptor A7 is required to generate CS mRNA for Tat, Rev, and Nef. This process is dependent on the complex interplay between exon splice silencer (ESS) and exon splice enhancer (ESE) elements located downstream of the A7 splice acceptor site. Both the ESS and ESE modulate the binding of U2AF, a subunit of the U2 snRNP, to the PPyT, which stabilizes the U2 snRNP binding to the 3'ss and allows splicing to occur.48 Because
U1 snRNP binding proximal to a 3’ss has been demonstrated to increase the recruitment of U2AF to the PPyT, it is possible that U1-Env acts through this mechanism. A similar mechanism is likely responsible for the effects of U1-Rev, which binds in proximity to U1-Env and was also shown to enhance splicing by increasing the inclusion of exon 4 in IS mRNA (Figure 5C). However, U1-Rev induced a general reduction in viral RNA species on northern blot (Figure 5A), suggesting that the inhibition seen is mainly mediated through the inhibition of polyadenylation. Our results demonstrate that U1i RNAs can both enhance splicing and inhibit polyadenylation but that one phenotype is dominant depending on the target site. Since the U1-Env target site is downstream of the U1-Rev target site and it had a more dominant splicing phenotype (Figure 5), our results also highlight that it is not just the proximity to the polyadenylation signal that dictates which phenotype is dominant.

Expression of U1-Gag2 resulted in a general reduction of viral RNA species as demonstrated by northern blot and RT-PCR gel (Figures 5A and 5C). These results demonstrate that U1-Gag2 does not act through enhancing HIV-1 RNA splicing. While it is possible that U1-Gag2 could be acting through inhibition of polyadenylation, the fact that it was not dependent on binding of the U1-A protein to the SL2 domain, whereas both polyadenylation inhibitors, U1-Rev and U1-Nef, were, suggests that it is acting through a distinct mechanism. Instead of enhancing splicing, our results are compatible with U1-Gag2 inhibiting splicing at the upstream 5’ss. This would lead to a reduction in all splice variants, including those coding for Tat mRNA. The lack of Tat expression would result in poor transcription of the FL RNA and subsequent decrease in all viral RNA isoforms. Although U1 snRNAs usually have a positive effect on splicing, there are several examples in the literature where they have been shown to negatively regulate splicing. For example, binding of the U1 snRNA to a pseudo-5’ss in the Gag coding sequence of Rous sarcoma virus results in non-productive interactions with the 3’ss that inhibit splicing at the upstream 5’ss. Other examples include U1 snRNA-mediated inhibition of pseudo-exon inclusion in the ataxia telangiectasia mutated gene, as well as the growth hormone receptor gene. Lastly, U1-Gag2 could also be interfering with D1 splice site utilization by inhibiting the action of cellular splice enhancer factors. This hypothesis is compatible with intronic splicing enhancer sequences located in the Gag open reading frame that regulate D1 splice site usage. Further studies are planned to better understand the mechanism of action of U1-Gag2 and to identify new U1i RNAs targeting the Gag or Pol region of HIV-1 RNA with more potent activity against HIV-1 replication compared to U1-Gag2.

To evaluate whether U1i RNAs with different mechanisms of action can inhibit viral replication in T lymphocytes, Sup-T1 cells were transduced with U1i RNAs and infected with different amounts of HIV-1 NL4-3 virus (Figures 6A and 6B). Cells transduced with U1-Gag2, Rev, and Env supported HIV-1 replication with similar kinetics to cells transduced with the U1-WT and empty vector. Comparable results were reported in a previous study for three different U1i RNAs designed to inhibit HIV-1 polyadenylation. In contrast, cells transduced with U1i RNAs that enhance splicing (U1-T6 and U1-D2) restricted virus replication, and U1-T6-transduced cells inhibited viral replication for a longer time when compared to cells transduced with an shRNA targeting the tat/rev reading frame of HIV-1 RNA, currently in clinical trials (ClinicalTrials.gov: NCT01961063, NCT02337985, and NCT00569985) (Figure 6C). A possible explanation for the superior effects of U1i RNAs that enhance splicing could be related to faster kinetics for this mechanism of inhibition, whereby many more transcripts are targeted by a single U1 molecule compared to the other mechanisms of action. Indeed, splicing occurs fairly rapidly, usually co-transcriptionally, whereas a U1i RNA that inhibits polyadenylation may remain associated for a longer time with its target RNA. Although only two U1i RNAs were effective at restricting viral replication in T lymphocytes, there was a correlation between their potency at inhibiting HIV-1 production in HEK293T cells (Figure 3B) and the extent to which they were able to restrict viral replication in Sup-T1 cells (Figures 6A and 6B).

With a recognition domain of only 9–11 nt, the specificity of U1i RNAs remains a significant concern. It has been argued that the specificity of U1i RNAs is enhanced by the fact that they only target 3’ terminal exons and that they are very sensitive to target site occlusion by RNA secondary structures, which are enriched in the 3’ end of mRNAs. However, as highlighted by the splicing enhancers (U1-D2 and U1-T6) and the possible splicing inhibition mediated by U1-Gag2, they could also have off-target effects at different locations in a mRNA by various alternative mechanisms. Notably, U1i RNAs targeting splicing sites have a high probability to interfere with regular gene splicing due to similarities in splice site sequences. There are more than 300,000 5’ss found in the human genome, with the majority having modest complementarity with the U1 snRNA, with a mean of 6 out of 10 nt. Since little homology is required, designing U1i RNAs targeting 5’ss may result in significant off-target effects. To reduce the potential for off-target effects, U1i RNAs can be designed to enhance splicing by targeting regions upstream of 3’ss, as they will likely have lower homology to the endogenous U1 snRNA. Indeed, based on our off-target prediction (Table 2), U1-D2, which targets the HIV-1 D2 splice site, had more potential off-target matches (5,467) compared to U1-T6 (1,827), which targets a region upstream of a 3’ss. Nevertheless, the large number of potential targets is a major concern for toxicities related to U1i RNAs, regardless of their target site. For further advancement into clinical trials, deep transcriptomics will need to be done on any candidate molecule to determine and characterize the off-target effects in different cellular and animal models.

To increase the specificity of U1i RNAs, we elongated their recognition domains. As with any RNAi technology, we hypothesized that longer binding domains would result in more specific inhibition. One study has demonstrated that the recognition domain of U1i RNAs can be extended by up to 6 nt with little effect on the inhibitory action of the molecule. However, in another study, increasing the recognition domain of a U1i RNA by 1–5 nt greatly decreased or completely abolished its inhibitory activity. Nonetheless, both of
these studies demonstrated that the altered inhibition by extended U1i RNAs was not due to differential expression of these molecules. With the exception of U1-Gag2, we found that the recognition domain of all U1i RNAs evaluated could be increased without affecting their activity (Figure 7A; Figure S4). However, some permutations were deleterious with no obvious trend regarding length or sequence. The loss of activity in these U1i RNAs could be due to a decrease in the nuclear accumulation of the molecules, or to poor hybridization with the target site due to secondary structures either in the target site or within the U1 molecule itself. These results could explain the discrepancy in the literature, in which each study evaluated length variants of only one U1i RNA molecule. A recent study has shown that the first 2 nt (AU) of the U1 snRNA are critical for splicing and that permutations at these nucleotides significantly reduced splicing efficiency. However, splicing enhancers U1-D2 and U1-T6 3′ + 6 were effective inhibitors of HIV-1 production, despite not having AU as their first 2 nt (Table 2), and U1-T6 3′ + 6 was even superior to other U1-T6 variants that all began with AU (Figure 7A). Interestingly, the only polyadenylation inhibitor (U1-Env) that started with AU was also the only one that strongly enhanced splicing (Table 2). Further studies will need to be conducted to determine whether the addition of AU start sites can improve the activity of U1i RNAs that enhance splicing and whether it can confer the ability to enhance splicing to U1i RNAs designed to inhibit polyadenylation. Overall, several length and start site permutations should be tested to identify the best format for any particular target site.

We also analyzed whether U1i RNA molecules with increased lengths could tolerate nucleotide mismatches within their target site. Using U1-T6 as our candidate molecule, due to its high efficacy in both viral production and infection assay, we co-transfected HEK293T cells with the different U1-T6 length variants and two different HIV-1 molecular clones (MJ4 and ADA) that had mutations within or around the original 9-nt targeted sequence. Co-transfection of HIV-1 molecular clone MJ4, which has a mismatch mutation positioned at the 3rd nt of the target site, resulted in a notable reduction of U1-T6 antiviral activity (Figure 7B). Similarly, in another study, a single mismatch in positions 3–8 of the U1i RNA recognition domain that differ from GU completely abrogated the silencing activity of U1i RNAs that inhibit polyadenylation. In contrast, co-transfection of U1-T6 5′ + 3 or 5′ + 6 resulted in an increased inhibition of MJ4, demonstrating that intolerance to a single mismatch can be overcome with an elongated recognition domain perfectly matched to its target site. This feature of the elongated U1 molecules makes them more attractive for use in anti-HIV-1 gene therapy, as it would be harder for HIV-1 to develop resistance against them through a single nucleotide mutation. Alternatively, the introduction of two or more mismatches between either MJ4 or ADA and the 3′ elongated U1-T6 variants resulted in a drastic loss in potency (Figures 7B and 7C). These results suggest that molecules with elongated recognition domains can have increased specificity, as mutations within their elongated target site reduce their antiviral efficacy. Although the predicted perfect matches of U1-T6 length variants to human RNAs was drastically reduced with the extended lengths (Table 2), it is likely that these molecules could have off-target effects without perfect matches, and deep transcriptomics will be needed to fully characterize the effects on the human transcriptome. Overall, our results suggest that U1i RNAs with elongated recognition domains represent superior therapeutic molecules; however, further studies are required to compare their specificity in more detail.

In conclusion, we have demonstrated that U1i RNAs that enhance splicing are potent inhibitors of HIV-1 replication. Their distinct mechanism of action and potential to act synergistically with shRNAs make them very attractive candidates to be used in a combination gene therapy approach for HIV-1. While our results demonstrate that a U1i RNA has similar efficacy to a potent anti-HIV-1 shRNA, the short recognition domain of 9–11 nt, compared to 19–21 nt for shRNAs, makes U1i RNAs much more likely to have off-target effects on human transcripts. However, our results suggest that it is possible to increase the recognition domain of some very active U1i RNAs to a total of 16 nt and maintain their efficacy. From our results we propose U1-T6 with a 6-nt elongated 5′ end (U1-T6 5′ + 6) as the best candidate for further development. Future studies will focus on determining its long-term safety and toxicity as well as its synergistic or antagonistic potential when combined with other antiviral RNA technologies such as ribozymes, decoy RNAs, aptamers, and shRNAs.

**MATERIALS AND METHODS**

**Plasmid Constructs**

UBC plasmids expressing WT and HIV-1-specific U1 snRNAs (U1-Rev and U1-Env) were provided by Sajic et al. All other HIV-1-specific U1 snRNA plasmids were created using PCR mutagenesis on the WT UBC plasmid (U1-WT). The 3′ (mutagenic) primers used to create the various U1i RNAs are shown in Table 1. The 3′ primer was 5′- AGTGCCAAGCTTGATGCGGAGTC-3′. A base change (underlined) was introduced into the 3′ primer to remove a PstI site found in the U1-WT plasmid and allow for PstI digestion discrimination against WT plasmids. PCR products and the U1-WT plasmid were digested with restriction enzymes BglII and HindIII to create the various U1i RNA plasmids. PCR products and the U1-WT plasmid to replace the U1 snRNA sequence.

U1i RNAs are shown in Table S1. To generate the UBC-empty plasmid, a short double-stranded scrambled sequence was made by annealing oligonucleotides 5′- GATCCCCGTCTCAAGCTGAGATCGAGATCGAGAAGTC-3′ with overhangs matching BgIII and HindIII cut sites. This was then ligated into BgIII and HindIII digested UBC-WT plasmid to replace the U1 snRNA sequence.

U1 snRNA SL1 and SL2 mutants (SL1Mut, SL2Mut), described in Sajic et al., were generated with the Q5 site-directed mutagenesis kit (New England Biolabs) using the primer pairs provided in Table S2. For SL1Muts, site-directed mutagenesis was performed on the individual U1 plasmids, while for SL2Muts, site-directed mutagenesis...
was performed on the U1-WT plasmid and the various U1 plasmids were cloned using PCR mutagenesis, as described above. For all plasmids, correct construction was confirmed by sequencing, using forward (5'-CCGAGTCAGGAGGTGGTAAGACG-3') and reverse (5'-AGGGATACAAATTTCCACAGG-3') primers.

**Cells and Transfections**

HEK293T cells (ATCC) were maintained in DMEM with high glucose (HyClone) supplemented with 10% fetal bovine serum (FBS) (HyClone), 50 U/mL penicillin, and 50 μg/mL streptomycin (Life Technologies). For transfections of U1i RNA expression plasmids, cells were seeded at 2.25 × 10⁵ cells/mL. 24 h prior to transfection in 96-, 24-, 12-, or 6-well plates in a volume of 150, 500, 1,000, or 2,000 μL, respectively. For production of HIV-1 NL4-3 virus or lentiviruses, cells were seeded at 2.25 × 10⁵ cells/mL. 24 h prior to transfection in T25 or T75 flasks, in a volume of 6 or 20 mL, respectively. Plasmid transfections were performed using TransIT-LT1 (Mirus) according to the manufacturer’s protocol. For all transfections, assays or viruses were harvested, 48 h after transfection. Supernatants were harvested in RPMI 1640 (HyClone) supplemented with 10% heat-inactivated (55°C, 30 min) FBS (HyClone), 50 U/mL penicillin, and 50 μg/mL streptomycin (Life Technologies).

**HIV-1 Production Assay**

Viral production was determined by measuring the activity of HIV-1 RT in the supernatant of co-transfected cells, as previously described.28,63 Briefly, HEK293T cells were co-transfected with 100 ng of HIV-1 molecular clones pNL4-3 (GenBank: M19921), pMJ4 (GenBank: AF321523), or pADA (GenBank: AF004394)28,65 and different amounts of U1i RNA plasmids or shRNA-expressing plasmids in 24- or 96-well plates. Forty-eight hours after co-transfection, 5 μL of supernatant was incubated for 2 h in 50 μL of RT cocktail (60 mM Tris-HCl, 75 mM KCl, 5 mM MgCl₂, 1.04 mM EDTA, 1% Nonidet P-40, 10 μg/mL poly(A), 0.33 μg/mL oligo(dT), 8 mM DTT, and [3²P]Deoxythymidine triphosphate [3,000 Ci/mmol]). Five microliters of the reaction mixture was then spotted onto a glass-fiber diethylaminoethyl filter mat (PerkinElmer) and left to dry for 10 min. Filter mats were washed five times for 5 min with 2× saline sodium citrate (SSC) buffer (20× SSC buffer: 3 M NaCl and 0.3 M sodium citrate), followed by two 1-min washes in 95% ethanol. Cpm were measured using a microplate scintillation counter (MicroBeta TriLux). RT data were normalized to cells co-transfected with negative control plasmids (U1-WT, U1-SDL1-WT, U1-SDL2-WT, psiRNA-H1-GFP-Zeo).

**Cell Viability Assay**

Cell viability was estimated by measuring the metabolism of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1; Sigma-Aldrich) to formazan (dark red) in HEK293T cells transfected with U1 plasmids in 96-well plates.66 As a positive control for cell toxicity, 10 μL of 3% w/v H₂O₂ (Sigma-Aldrich) was added to control wells at the time of transfection. Forty-eight hours after transfection, 100 μL of cell culture media was removed from each well and 10 μL of WST-1 reagent was added. Cells were then incubated for 4 h at 37°C and 5% CO₂. Plates were read at 450 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad). All results were normalized to cells transfected with U1-WT.

**HIV-1 Sequence Conservation Estimates**

The QuickAlign tool on http://www.hiv.lanl.gov/content/index was used to estimate the conservation at the nucleotide level of sites targeted by U1i RNAs among circulating HIV-1 strains as previously described.27,28 Briefly, sequence alignments containing all complete HIV-1 genome sequences (3,666 at the time of analysis) were generated using the QuickAlign tool. Jalview alignment editor (version 2.9) was used to calculate the percentage conservation for each nucleotide in the selected target site expressed as the percentage of sequences containing the consensus nucleotide at each position. The QuickAlign tool was also used to generate a sequence logo for one target site.

**HIV-1 Intracellular RNA Expression**

HEK293T cells were co-transfected with 400 ng of HIV-1 pNL4-3 and 1,000 ng of U1 plasmids in six-well plates. Forty-eight hours after co-transfection, cell lysates were harvested in TRIzol (Invitrogen). Total RNA was isolated using phenol chloroform extraction followed by clean-up using RNeasy Mini Kits (QiAGEN). RNA (20 μg) was resolved on a 1% agarose gel (1 g of agarose in 84.6 mL of water), 10 mL of 10× MOPS buffer (200 mM 3-(N-morpholino)propanesulfonic acid [MOPS], 50 mM sodium acetate, 10 mM EDTA, 0.1% diethyl pyrocarbonate [DEPC], pH 7.0), 5.4 mL of 37% formaldehyde (EMD Millipore), and 10 μL of RedSafe and transferred to a Hybrid-N membrane (GE Healthcare Amersham). Equal loading was determined by UV visualization of the agarose gel prior to the transfer. Blots were hybridized with digoxigenin (DIG)-labeled DNA probes targeting the HIV-1 long terminal repeat (LTR) (generated by PCR using the primers 5'-CTAATTACATCCTAAAAGAGA-3' and 5'-TGCTAGAGATTTTCCACACTG-3'). DIG labeling, hybridization, washes, and visualization of RNA were performed as in the DIG DNA Labeling and Detection Kit protocol (Roche).

**RT-PCR Analysis of Viral RNA Species**

Total RNA was isolated as indicated above and treated with DNase I for RT-PCR analysis.67 1 μg of DNase-treated RNA was used for cDNA synthesis using a ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs). Viral cDNA was subsequently amplified using forward primer 5'-CTGAGCTGGAGCTCTTGCC3'- and reverse primer 5'-TCATTGCCACCTGTCTGCTC3'- for 4-kb viral RNA and forward primer 5'-CTGAGCTGGAGCTCTTGCC3'- and reverse primer 5'-CCGACAGATGCAGCCAGAAGAGA-3' for 2-kb viral RNAs. The PCR products were resolved on a 3% agarose gel (25 μL of RedSafe) and visualized with UV light.

**HIV-1 Infection and Competitive Growth Assays**

U1i RNA expression cassettes from this study and shRNA expression cassettes generated in our previous study27 were subcloned into the HIV-7-EGFP plasmid (donated by Dr. J. Rossi).57 Lentiviral vectors were produced by co-transfecting HEK293T cells with the HIV-7-
EGFP plasmids, a vesicular stomatitis virus G protein (VSV-G) expression plasmid (from Dr. J. Rossi), and a second-generation packaging plasmid (pSPAX2, Addgene, no. 12260). The supernatants were collected 48 h later and the lentiviral particles were concentrated using Lenti-X (Clontech) according to the manufacturer’s protocol. Lentiviruses were titered on Sup-T1 cells using percentage GFP-positive cells, and an MOI of 3.5 was used to transduce 5-mL cultures of Sup-T1 cells at 3.5 × 10⁵ cells/mL with 8 μg/mL Polybrene (Sigma-Aldrich). Seventy-two hours after transduction, cells were sorted for GFP expression using the gates shown in Figure S3. For one set of transduced cells (Figure S3A), cells were immediately plated in a 96-well round-bottom plate at 1 × 10⁵ cells/well and infected with HIV-1 NL4-3 24 h later in triplicate at 8,750 cpm/mL and 1,750 cpm/mL, measured using the HIV-1 RT assay. For the other set of transduced cells (Figure S3B), cells were plated 24 h after sorting or frozen and stored at −80°C. For the first infection, cells were plated at 1 × 10⁵ cells/well and infected with HIV-1 NL4-3 virus in triplicate at 8,750 cpm/mL. For the second infection, cells were defrosted, cultured for 72 h, and infected as in the first infection. HIV-1 replication was monitored by measuring HIV-1 RT activity in cultured supernatants of infected cells. For the competitive growth assay, the second set of transduced cells (Figure S3B) was plated at 5 × 10⁵ cells/well with non-transduced Sup-T1 cells, also at 5 × 10⁵ cells/well in quadruplicate. The cultures were passed two times a week by removing 100 μL of media and cells and adding 100–110 μL of fresh media. The percentage of GFP-positive cells in the cultures was measured at different times after the cells were plated.

Statistical Analysis
All results with repeated measurements are expressed as the mean ± SEM. To determine statistical significance for dose-response data (Figures 2B, 3B, and 7) and for infection time course data (Figure 6), a two-way ANOVA with a Bonferroni post-test was used to compare replicate means to the means of U1-WT or empty shRNA-expressing vector-transfected cells (Figures 2B, 3B, and 7) or U1-WT-transduced cells (Figure 6). For single-dose data (Figure S2), unpaired two-tailed t tests were used to compare the means of each treatment group to U1-WT-transfected cells. All statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad).

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
A.G. and R.J.S. conceived the study. R.J.S. and O.D.C. designed and conducted most of the experiments and wrote the manuscript. A.G. revised the manuscript. R.P.G. and C.M.G.M. participated in study design and execution. A.D. and S.C.-G. assisted in conducting experiments. All authors read and approved the manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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