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Inhibition of Human Immunodeficiency Virus Type 1 Replication with Artificial Transcription Factors Targeting the Highly Conserved Primer-Binding Site

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The human immunodeficiency virus type 1 (HIV-1) primer-binding site (PBS) is a highly conserved region in the HIV genome and represents an attractive target for the development of new anti-HIV therapies. In this study, we designed four artificial zinc finger transcription factors to bind at or adjacent to the PBS and repress transcription from the HIV-1 long terminal repeat (LTR). These proteins bound to the LTR in vivo, as demonstrated by the chromatin immunoprecipitation assay. In transient reporter assays, three of the four proteins repressed transcription of a reporter driven by the HIV-1 LTR. Only one of these proteins, however, designated KRAB-PBS2, was able to prevent virus production when transduced into primary lymphocytes. We observed >90% inhibition of viral replication over the course of several weeks compared to untransduced cells, and no significant cytotoxicity was observed. Long-term exposure of HIV-1 to KRAB-PBS2 induced mutations in the HIV-1 PBS that reduced the effectiveness of the repressor, but these mutations also resulted in decreased rates of viral replication. These results show that KRAB-PBS2 has the potential to be used in antiviral therapy for AIDS patients and might complement other gene-based strategies.

AIDS is a viral immune system disorder that has reached pandemic proportions in the last several decades. The virus responsible for the disease, human immunodeficiency virus (HIV), infects $CD4^+$ T cells and establishes a latent pool of infected cells. Current treatment for AIDS involves the use of a multidrug cocktail referred to as highly active antiretroviral therapy (HAART). While HAART has proven to be a potent treatment for the disease, there are significant drawbacks to this approach, including toxicity, numerous side effects, and more importantly, mutation of the virus to escape the effects of the cocktail (21). Furthermore, despite initial decreases in viral load immediately following HAART, a reservoir of latently infected cells remain in the patient's blood and virus is rapidly reactivated following drug withdrawal (48). Thus, new methods need to be developed to counteract the ability of HIV to escape therapy by mutation and also to prevent latently infected cells from replenishing viral titers in the patient. While HIV-1 does have a high mutation rate, certain sequences in the viral genome must be conserved for proper replication of the virus, and targeting these sequences for the development of new therapies is the goal of many researchers.

The virus life cycle consists of a number of steps that require both virus and host factors. Interruption of any one of these steps would provide a viable means for inhibiting virus production. One particularly well-studied step of the HIV-1 life cycle is transcription of RNA from the integrated viral genome. The HIV-1 5′ long terminal repeat (LTR) contains binding sites for a number of host transcription factors, including Sp1, NF-κB, and Lef-1 (43). Binding of these factors mediates initiation of transcription by RNA polymerase II, and binding of the HIV-1 Tat protein to the *trans*-activating response element on the nascent transcript stimulates elongation by recruitment of a host protein, positive transcription elongation factor b (37). A number of strategies have been employed to target HIV-1 transcription including ribozymes (19, 59), antisense oligonucleotides (2), and more recently, RNA interference (RNAi) (8, 25, 26). One drawback of these approaches is that they are designed to target mRNA; thus, multiple copies of the target must be inactivated. Highly expressed mRNAs may be difficult to completely repress, as most viral transcripts are. Therefore, a more efficient strategy is to target transcription at the DNA level, as only one DNA provirus must be bound to inhibit virus production.

The C_2H_2 zinc finger (ZF) motif is the most ubiquitous DNA-binding motif in metazoans. A single zinc finger consists of a simple ββα fold coordinated by a zinc ion; residues in the α-helix make specific contacts with three nucleotides. Previous work in our laboratory has led to the identification of domains that recognize the 5′-(G/A/C)NN-3′ subsets of the 64-member triplet alphabet (16, 17, 49) In addition, many domains recognizing the 5′-TNN-3′ family of sequences have been characterized. The modularity of the zinc finger motif allows the construction of polydactyl transcription factors that potentially bind to unique sites in a genome. Appending a transcriptional activator, such as the VP16 transactivation domain (47), or a

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repressor, such as the Kruppel-associated box (KRAB) domain (38), allows potent up- or down-regulation of a gene of interest (3–5, 34). Such artificial transcription factors have been shown to regulate numerous endogenous genes in many different animal and plant cells (3, 22, 23, 28, 53, 61).

We have previously used artificial zinc finger transcription factors to regulate the HIV-1 5′ LTR (50). One of the proteins tested, designated HLTR3, effectively inhibits HIV-1 transcription after transient transfection and inhibits viral replication in cell lines and in peripheral blood mononuclear cells (PBMCs) that stably express the transcription factor. HLTR3 binds to a site in the HIV-1 LTR that overlaps two Sp1-binding sites. Other studies have also targeted this region with ZF transcription factors (30, 45). This sequence is well conserved in the B clade, but this region shows significant sequence variation in some other clades (27). Thus, we sought to target a sequence in the LTR that is well conserved across all clades. The tRNA primer-binding site (PBS) is the most highly conserved site in the HIV-1 genome. Human $tRNA_3^{Lys}$ binds to the PBS and is used as a primer for reverse transcription (36, 39). The PBS is completely conserved across clades (14), and mutations to the PBS negatively affect virus production and infectivity (42, 46). Sequences flanking the PBS are also highly conserved. Furthermore, the PBS is located at the 3′ end of the LTR in a nucleosome-free sequence that is accessible to DNase I and micrococcal nuclease digestion (54); therefore, a ZF protein targeted to this site should bind and regulate transcription.

In this study, several ZF proteins were designed to bind to sequences within the HIV-1 PBS and flanking sequences and were fused to the KRAB repression domain. These transcription factors were tested for their ability to repress transcription of the LTR in reporter assays and to inhibit virus production after infection of PBMCs. Finally, an escape assay was performed to determine if long-term exposure to the ZF would induce mutation of the virus that could reduce the effectiveness of the repressor. Our results suggest transcriptional repressors that target the HIV-1 PBS are potential new therapeutics for HIV-1 disease.

MATERIALS AND METHODS

Construction of custom DNA-binding proteins. DNA-binding proteins containing six zinc finger domains were assembled onto an Sp1C zinc finger scaffold using methods and domains described previously (4, 16, 18, 49). Briefly, overlapping PCR primers were designed to encode zinc finger domains that had been previously determined to bind unique 3-bp sites. Three-finger proteins were assembled by overlap PCR then assembled into six-finger proteins by Age I/XmaI ligation. Recently, we released a web-based program called Zinc Finger Tools that allows zinc finger proteins to be automatically designed (http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php). For in vitro characterization, the constructs were cloned into the prokaryotic expression vector pMAL-c2 (New England Biolabs). Fusions with the maltose-binding protein were expressed and purified with the Protein Fusion and Purification system (New England Biolabs). Electrophoretic mobility shift assays were performed as described previously (4, 49).

Effector and reporter plasmids. The PBS1, PBS1a, PBS2, and PBS3 six-finger proteins were cloned into a pcDNA-based effector plasmid containing the KRAB repression domain as described previously (4). The four six-finger proteins were also cloned into the pMX retroviral and pSIN lentiviral vectors by digesting pMX KRAB-HLTR3 and pSIN-KRAB-HLTR3 (50) with SfiI and then ligating each of the four six-finger proteins digested with SfiI from the pMal-c2 vectors with the pMX retroviral and pSIN lentiviral vectors. The HIV-1 LTR reporter, LTR658-luc, was constructed by amplifying the HIV LTR by PCR from the plasmid pIIIenv3-1 (National Institutes of Health AIDS Research and Reference Reagent Program [NARRRP]) (52) using the forward primer 5′-GATACGACA GCTAGCTGGAAGGGCTAATTCACTCCC-3′ and the reverse primer 5′-AAC GTCTGGCTCGAGTTCAGGTCCCTGTTCGGGCGCCACTGCTAGAGATTT TCC-3′. The PCR product was digested with NheI and XhoI and was ligated into the pGL3 control vector (Promega) previously digested with NheI and XhoI. The pGL3 promoter vector (Promega), driven by the simian virus 40 (SV40) promoter, was used as a negative control.

Cell culture and transient transfection assays. HeLa cells (American Type Culture Collection), TZM-bl cells (NARRRP) (15, 44, 60), and Gag-Pol 293 cells (Clontech) were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin-antimycotic (Gibco). For transient transfection assays, approximately 5×10^4 cells were seeded into 24-well plates to 40 to 60% confluence. HeLa cells were transfected with 10 ng of reporter plasmid, 75 ng of the Tat-expressing plasmid pSV2tat72 (NARRRP) (20), 100 ng of effector plasmid, and 100 ng of CMV-lacZ plasmid using Lipofectamine transfection reagent (Invitrogen). TZM-bl cells were transfected similarly, except that no reporter plasmid was used and 50 ng of the *Renilla* luciferase plasmid pRL-CMV (Promega) was transfected in place of CMV-lacZ. Cell extracts were prepared ~48 h after transfection. Luciferase and β-galactosidase activities were measured using assay reagent kits from Promega and Tropix, respectively, in a MicroLumat LB96P luminometer (EG&G Berthold, Gaithersbug, MD). Luciferase activity was normalized to β-galactosidase activity in HeLa cells and to *Renilla* luciferase activity in TZM-bl cells. Determination of ZF protein expression and inhibition of transiently transfected, plasmid-based HIV expression were performed as described previously (50).

Retroviral delivery of ZF proteins and chromatin immunoprecipitation (ChIP). Retroviral transductions of the KRAB-PBS proteins into TZM-bl cells were performed using the Moloney murine leukemia virus-based pMX vector, essentially as described previously (35). Transduction efficiency was monitored by flow cytometric analysis of green fluorescent protein expressed via an internal ribosome entry site within the zinc finger expression cassette (data not shown). Approximately 10^7 infected cells were cross-linked with 1% formaldehyde, and chromatin was prepared as described previously (7). Chromatin was immunoprecipitated with 1 μg RNA polymerase II antibody (Santa Cruz Biotechnology), 10 μl ZF antibody (35), or no antibody. Immunoprecipitated chromatin was washed extensively and DNA was purified as described previously. Immunoprecipitated DNA, as well as a 1:100 dilution of 20% of the total input DNA taken from the no-antibody samples, was amplified by 30 to 35 cycles of PCR using the HIV-1 LTR primers 5′-CCG CTG GGG ACT TTC CAG GGA-3′ and 5′-CAC TGC TAG AGA TTT TCC ACA CTG-3′.

Northern blot analysis of tRNA expression. HeLa cells were plated at a density of 2×10^6 cells on a 10-cm dish, and cells were transfected with 4 μg of plasmid expressing the indicated KRAB-ZF or empty plasmid as a negative control. After 48 h, total RNA was isolated using TRIzol Reagent (Invitrogen). A total of 60 μg of RNA was separated on a 15% Tris-borate-EDTA–urea polyacrylamide gel and transferred to a GeneScreen Plus membrane (Perkin-Elmer) with an XCell II Blot Module (Invitrogen) according to the manufacturer's instructions. Prehybridization of the membrane was done in 5 ml of Rapid-hyb buffer (Amersham Biosciences) at 42°C for 15 min. The membrane was then hybridized with 10^6 to 10^7 cpm of a ^{32}P-end-labeled tRNA probe for 1 to 2 h at 42°C. The blot was washed twice for 15 min at 42°C in 0.1% SDS–0.1× SSC (15 mM NaCl and 1.5 mM sodium citrate) and then exposed 24 to 48 h on a phosphor screen, which was developed with a Molecular Dynamics phosphorimager. The sequence of the $tRNA_3^{Lys}$ probe is 5′-CGCCCGAACAGGGAC-3′, and the $tRNA^{Phe}$ probe is 5′-TGCCGAAACCCGGGA-3′.

Lentiviral production and delivery and HIV-1 infection of PBMC. Lentiviral production, titration, transduction into PBMCs, and subsequent challenge with HIV-1 were performed with a self-inactivating lentiviral vector system as described previously (50). The in vitro selection of HIV-1 clones resistant to PBS zinc fingers was performed as described by Keulen et al. (29). Briefly, selection was initiated by infection of SupT1 cells with wild-type NL4-3 (multiplicity of infection [MOI] of 0.02). After 1 h at 37°C, the culture was split, and cells were transduced with each zinc finger repressor at an MOI of 1 or 10. An assay of nontransduced cells was used to determine the levels of viral replication and cell growth in the absence of inhibitory zinc finger. Cells were transduced a second time at day 5. At day 5, after the second transduction, half of the cultures were used for titration in a 96-well plate, by limiting dilution as described by Keulen et al. (29). After 1 week, samples from individual wells were transferred to 24-well plates to optimize cell growth and viral replication. Cell-free supernatant and cells were harvested from individual wells when large syncytia were observed. Genotypic analysis of cells was performed with primers overlapping the

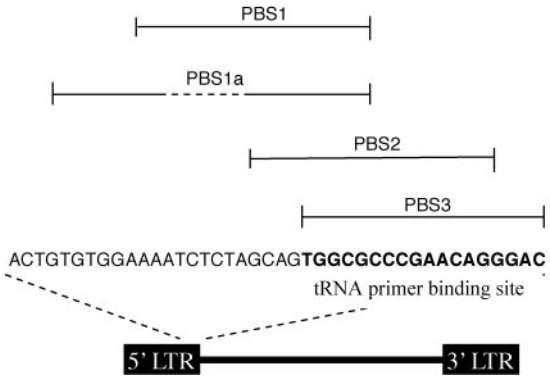

FIG. 1. Binding sites of PBS zinc finger proteins on the HIV-1 LTR. The sequence shown is from an HXB2 reference strain.

PBS region. PCR products were cloned into a TA cloning vector (pGEM-Teasy; Promega), and individual clones were sequenced. After 2 to 3 weeks of culture, 20 wells were considered positive by comparison with viral replication in non-expressing zinc finger cells. No positive wells were obtained after titration of cells transduced with an MOI of 10. It is possible that wild-type virus was able to replicate in some of the wells with transduction at an MOI of 1. Therefore, the in vitro-selected viruses were passaged onto fresh SupT1 cells previously transduced with SIN-PBSB at an MOI of 1. Ten samples were able to replicate optimally at an MOI of 1. Sequence analysis of these 10 viruses revealed the presence of a G-to-A mutation in the PBS site.

RESULTS

Design, expression, and analysis of primer-binding site zinc finger proteins. Four zinc finger proteins were constructed to bind to the HIV-1 PBS (Fig. 1; Table 1). Three of the proteins were designed to bind to 18 nucleotide sequences just upstream or within the PBS. One of the proteins, PBS1a, was designed to bind to two nonadjacent 9-bp sites separated by 7 bp. It consisted of two three-finger domains separated by a long linker (GGG SGGGGEKP). As an initial assessment of their ability to regulate HIV-1 transcription, the binding constant of each of the ZFs to the PBS was determined. Proteins were expressed and purified as maltose-binding protein fusion proteins and then tested for the ability to bind an oligonucleotide hairpin containing the PBS sequence in multitarget enzyme-linked immunosorbent assays and electrophoretic mobility shift assays (EMSAs) as described previously (50). The equilibrium dissociation constant (K_D) values of the proteins PBS1, PBS1a, PBS2, and PBS3 for their targets were 0.79, 1.25, 2.4, and 0.93 nM, respectively, as determined by EMSA (Fig. 2A).

To enable repression of transcription, the ZF proteins were fused to the KRAB domain. The proteins were then expressed in HeLa cells, along with LTR658-luc, a luciferase reporter driven by the HIV-1 LTR, and a construct expressing the Tat protein. As a negative control, a six-finger protein that does not bind the HIV-1 LTR but is functional in regulating reporters containing its target sequence, KRAB-Aart, was also tested. KRAB-PBS1, KRAB-PBS1a, and KRAB-PBS2 repressed expression of the reporter by 20, 9.5, and 28 fold, respectively. No significant repression was observed for KRAB-PBS3; as expected, KRAB-Aart did not repress the reporter. In addition, none of the PBS zinc fingers expressed with the addition of the repression domain altered expression of a luciferase reporter driven by the SV40 promoter (Fig. 2B). Finally, to ensure that these observations were not due to differences in protein expression, Western blot analysis was performed. KRAB-PBS2 and KRAB-PBS3 were expressed at ~2-fold-higher levels than KRAB-PBS1 and KRAB-PBS1a (Fig. 2C).

Repression of HIV-1 LTR expression in chromatin by PBS ZFs. In a transient transfection, a reporter plasmid is not packaged into chromatin in the same manner as a chromosomal gene, and so regulation seen in a transient assay may not be observed with a genomic target. It is a more relevant assay to test the ability of the PBS-binding ZF proteins to repress a reporter integrated into a mammalian genome. Thus, the transient transfection of the PBS ZFs and Tat was repeated in the TZM-bl cell line, a HeLa derivative that contains integrated copies of a lentivirus-based luciferase reporter and a β-galactosidase reporter (Fig. 3A, top). Repression of the HIV-1 LTR was observed in the presence of KRAB-PBS1, KRAB-PBS1a, and KRAB-PBS2, while KRAB-PBS3 and KRAB-Aart showed no repression, as was observed when the reporter was transiently transfected (Fig. 3A). The ZFs repressed the chromosomally integrated reporters at levels similar to those observed in the transient assay. The luciferase reporter was repressed 7 fold by KRAB-PBS1, 15 fold by KRAB-PBS1a, and 20 fold by KRAB-PBS2. Similar levels of repression were also observed for the β-galactosidase reporter.

To ensure that the PBS ZFs bound to the HIV-1 LTR, the ChIP assay was performed with TZM-bl cells. The PBS ZFs were expressed in TZM-bl cells by retroviral infection. The cells were cross-linked with formaldehyde, and chromatin was

TABLE 1. ZF sequences assembled to regulate the HIV LTR; the predicted 18-bp DNA-binding sites are indicated

TF_{SZF}	ZF helix						Predicted target site[c]
	F6	F5	F4	F3	F2	F1	Half site 1–half site 2
PBS1[a]	QRANLRA	RGGWLQA	QRHSLTE	QSGDLRR	RSDVLVR	RSDDLVR	5′-AAA TCT CTA–GCA GTG GCG-3′
PBS1a[b]	RSDVLVR	RSDHLTT	QRANLRA	QSGDLRR	RSDVLVR	RSDDLVR	5′-GTG TGG AAA atctcta GCA GTG GCG-3′
PBS2	QSGDLRR	RSDVLVR	RSDDLVR	HTGHLLE	QSSNLVR	RADNLTE	5′-GCA GTG GCG–CCC GAA CAG-3′
PBS3	RSDHLTT	HTGHLLE	RNDTLTE	DSGNLRV	RSDHLTN	DPGNLVR	5′-TGG CGC CCG–AAC AGG GAC-3′

[a] ZF helices are positioned in the antiparallel orientation (COOH-F6 to F1-NH2) relative to the DNA target sequence. Amino acid positions −1 to +6 of each DNA recognition sequence are shown. Web-based software is available for automated zinc finger protein design (http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php).

[b] PBS1a is composed of two three-finger ZFs separated by a long flexible linker ($Gly_3SerGly_4$). F3 to F1 bind to the second half site, GCA GTG GCG, and F6 to F4 bind to the first half site, GTG TGG AAA. The intervening nonbound DNA sequence is indicated by lowercase letters.

[c] Predicted target DNA sequences are presented in the 5′ to 3′ orientation.

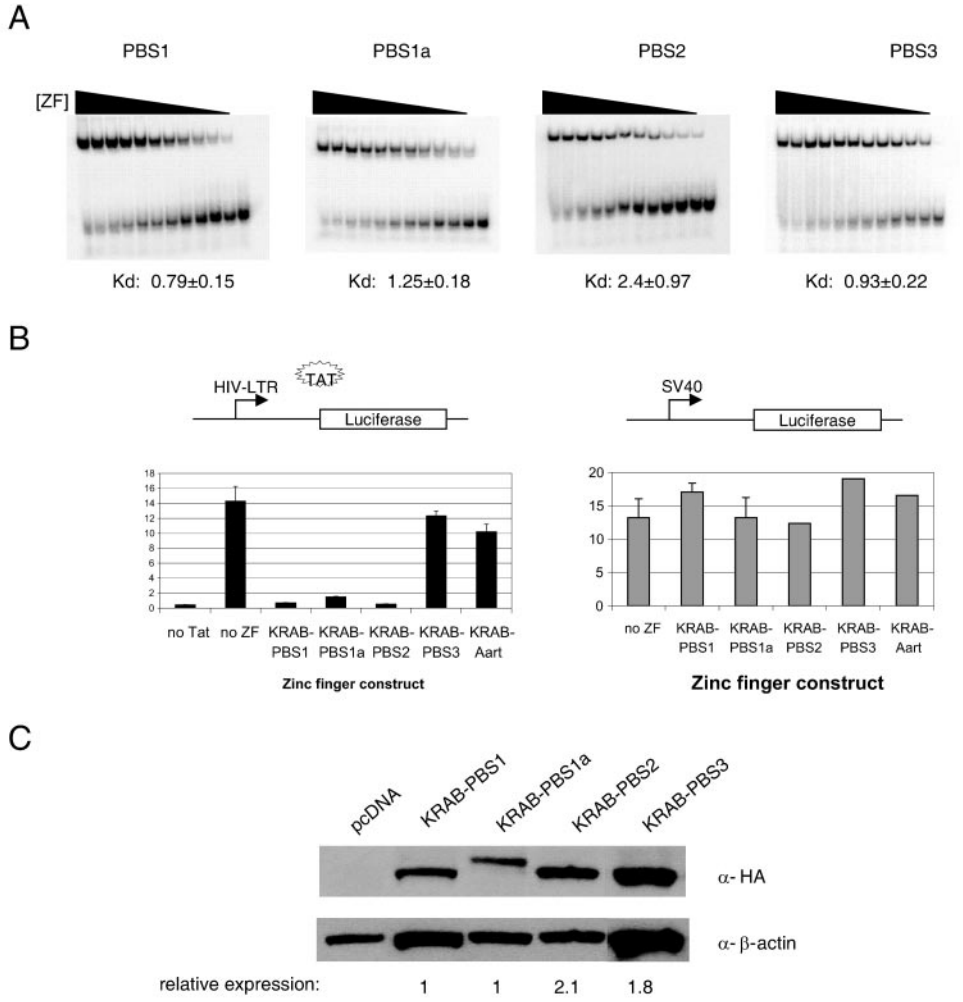

FIG. 2. PBS zinc finger-binding constants and inhibition of HIV-1 LTR in a transient transfection assay. (A) Gel shifts of the PBS zinc fingers. A ^{32}P-labeled DNA hairpin oligonucleotide containing the PBS sequence was incubated with decreasing amounts of protein. The density of free and bound DNA was quantitated using ImageQuant software, and the K_D for each protein was calculated. (B) Transient reporter assays comparing repression of the PBS proteins fused to the KRAB repression domains. The graph on the left shows results from transfection of KRAB-zinc finger proteins with an HIV-1 LTR-driven luciferase reporter and a plasmid expressing the Tat protein. The graph on the right shows control transfection of KRAB-zinc finger proteins with an SV40 promoter-driven luciferase reporter. (C) Protein expression levels of PBS zinc finger proteins. Cells were transiently transfected with zinc finger proteins as in panel B, and cell extracts were prepared. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were probed with an antibody recognizing the HA tag on the zinc finger proteins or a β-actin antibody. Relative protein expression was calculated by normalizing zinc finger expression to β-actin expression in each sample.

sonicated and isolated from the nuclei. ZFs bound to DNA were immunoprecipitated with an antibody that recognizes the zinc finger backbone, and immunoprecipitated DNA was analyzed by PCR using primers specific for the HIV-1 LTR. Chromatin was immunoprecipitated with a RNA polymerase II antibody as a positive control. In agreement with the reporter assays, we found that KRAB-PBS1, KRAB-PBS1a, and KRAB-PBS2 were bound to the HIV-1 LTR, whereas KRAB-PBS3 did not bind to the LTR in vivo (Fig. 3B). Because the primer-binding site is complementary to $tRNA_3^{Lys}$, it is possible that KRAB-PBS3 could bind to the $tRNA_3^{Lys}$ gene and to the HIV LTR and repress expression of the tRNA, since the KRAB domain has been shown to affect RNA polymerase III transcription (41, 51). To test this possibility, a Northern blot was performed to measure the levels of $tRNA_3^{Lys}$ in the presence of KRAB-PBS2, KRAB-PBS3, or KRAB-Aart. After normalization to $tRNA^{Phe}$ was performed, we found no significant effect on $tRNA_3^{Lys}$ levels from any of the ZF proteins (Fig. 3C).

Inhibition of HIV-1 virus production by PBS ZFs. As a preliminary test of the ability of the PBS ZFs to inhibit HIV-1 virus production under conditions of an infection, each of the KRAB-PBS expression plasmids was cotransfected with a plasmid encoding the HIV-1 strain NL4-3 genome. After 48 h, significant inhibition of virus production was seen in cells expressing KRAB-PBS1 (~75%) and KRAB-PBS2 (~90%), whereas KRAB-PBS1a and KRAB-PBS3 inhibited virus production by <50% (Fig. 4).

We next explored whether the transcription factors could

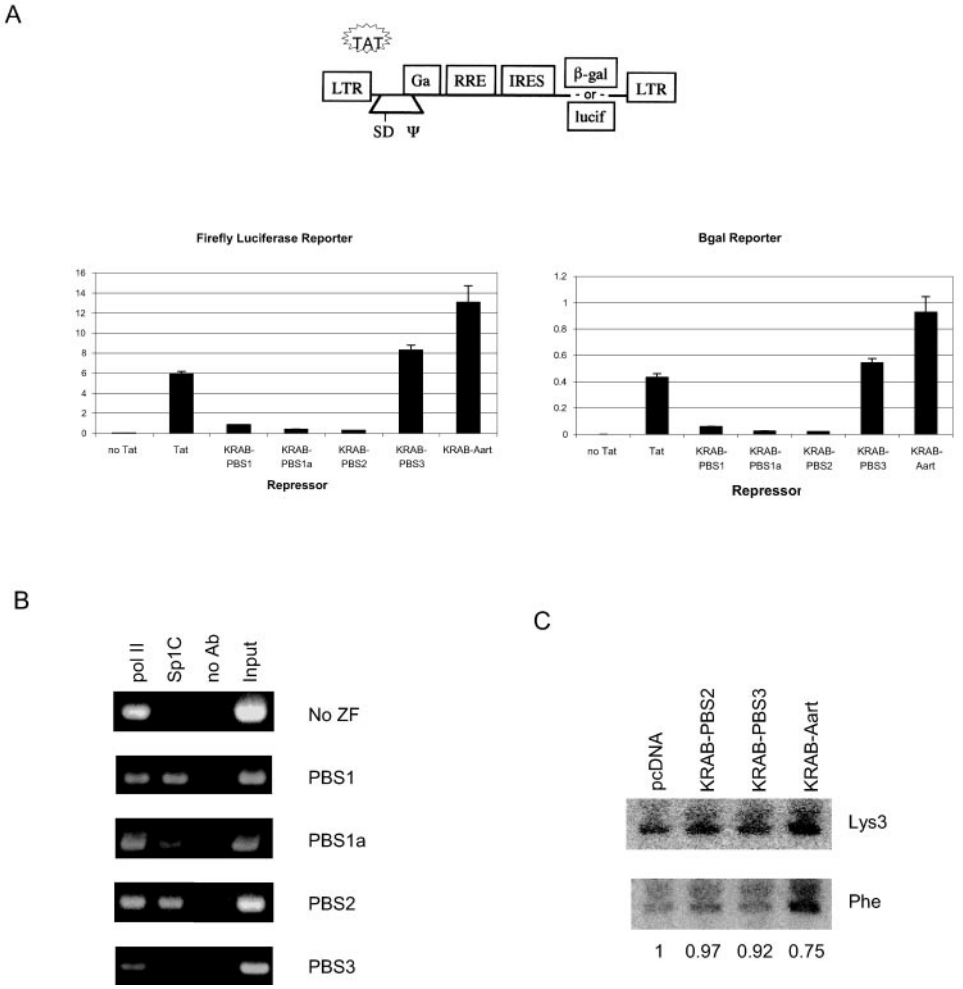

FIG. 3. Repression and in vivo binding of PBS zinc finger proteins on a chromosomally integrated HIV-1 LTR reporter. (A) KRAB-PBS zinc finger proteins and Tat were transiently expressed in the TZM-bl cell line, a HeLa cell line containing chromosomally integrated HIV constructs that drive expression of luciferase and *lacZ* genes. (B) Chromatin immunoprecipitation of zinc finger protein-bound HIV-1 LTR. Zinc finger proteins were expressed by retroviral transduction in TZM-bl cells. Cells were cross-linked with formaldehyde, and nuclear extract was prepared. The extract was incubated with RNA polymerase II (pol II) or an antibody recognizing an Sp1 consensus zinc finger (Sp1C) and precipitated with Staph A cells. Immunoprecipitated DNA was purified and analyzed by PCR for the presence of the HIV-1 LTR using primers specific for the LTR. (C) Northern blot of $tRNA_3^{Lys}$. HeLa cells were transfected with the indicated KRAB-ZF proteins, and total RNA was extracted. RNA was separated by denaturing polyacrylamide gels and transferred to a membrane, which was probed with radiolabeled oligonucleotides for $tRNA_3^{Lys}$ and $tRNA^{Phe}$. The numbers below each lane represent the relative expression of $tRNA_3^{Lys}$ after normalization to $tRNA^{Phe}$.

inhibit virus production in a more physiological setting. Therefore, we analyzed the effect of the PBS ZFs on viral replication in primary T cells infected with HIV-1. PBMCs were transduced with lentiviral vectors that expressed the KRAB-PBS transcription factors. Flow cytometry analysis using an antibody that recognizes the C-terminal hemagglutinin (HA) tag of the ZFs was performed to determine the efficiency of transduction; approximately 20 to 30% of the PBMCs expressed the ZFs (Fig. 5A). We also performed a flow cytometry analysis in which KRAB-PBS2-transduced cells were stained with a CD4 antibody in addition to the HA antibody, which showed that approximately two-thirds of the transduced cells were CD4-expressing and therefore HIV-1-permissive cells (Fig. 5A). These results agree with our previous results in transducing peripheral blood mononuclear cells (50). Transduced cells were challenged with HIV-1, and virus production was moni-

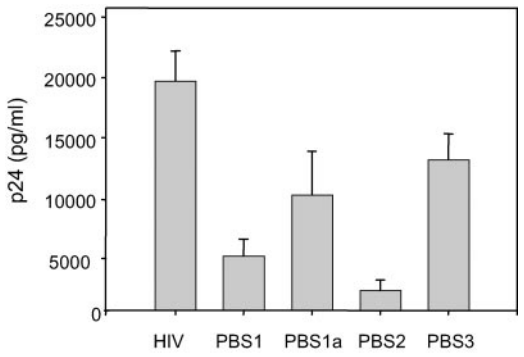

FIG. 4. Transient inhibition of virus production by PBS zinc finger proteins. Plasmids expressing the indicated KRAB-PBS zinc finger and the genome of the NL4-3 strain of HIV-1 were cotransfected into 293T cells. Virus production was monitored by a p24 ELISA.

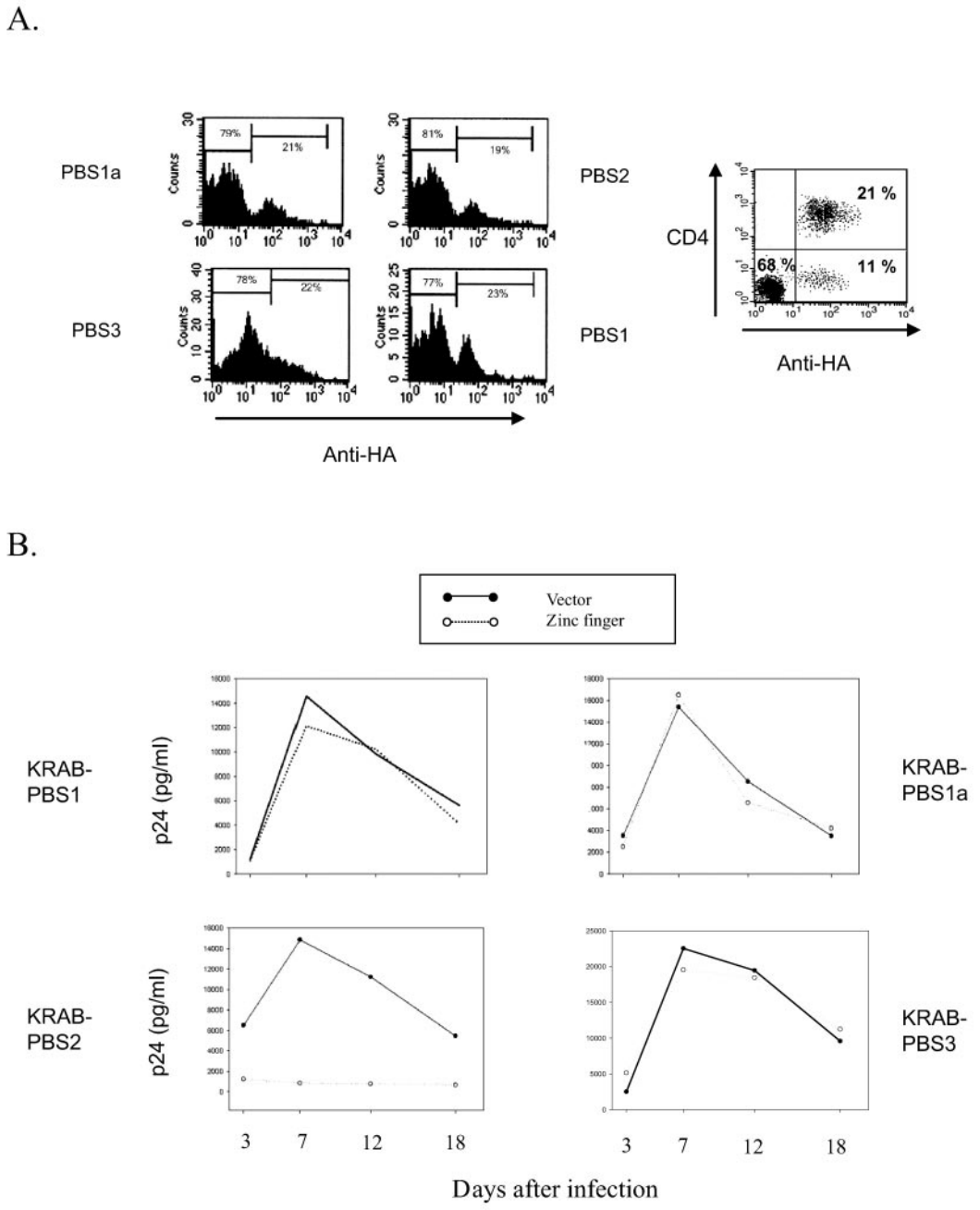

FIG. 5. Inhibition of HIV virus production by PBS zinc fingers in primary cells. (A) Flow cytometry analysis of zinc finger expression in PBMCs. PBMCs were transduced with lentiviral vectors that express KRAB-PBS zinc finger proteins. Cells were fixed and stained with an antibody that recognizes the C-terminal HA tag of the ZFs and analyzed by flow cytometry for zinc finger expression. The right panel shows flow cytometry analyses of KRAB-PBS2-transduced cells stained for CD4 and HA antibodies. (B) PBMCs were transduced with the indicated KRAB-PBS lentiviral vector and then challenged with infection by the HIV-1 strain NL4-3 at an MOI of 0.1. p24 levels were monitored at 3, 7, 12, and 18 days after infection.

tored by p24 quantitation. Of the four PBS-binding transcription factors, only KRAB-PBS2 was able to inhibit virus production. In cells transduced with KRAB-PBS2, a >90% reduction of virus production was observed, compared to that for nontransduced cells (Fig. 5B). To ensure that the reduced virus production was not due to cytotoxicity due to overexpression of KRAB-PBS2, cell viability was measured at various time points after HIV-1 infection. All samples were found to have similar viability at all time points measured (Fig. 6).

We previously found that KRAB-HLTR3, a protein designed to bind at a Sp1 site in the HIV-1 LTR, effectively inhibited viral replication. The HLTR3 ZF protein was designed to bind to the LTR of the B subtype of HIV-1, the predominant strain of the virus in the United States and Eu-

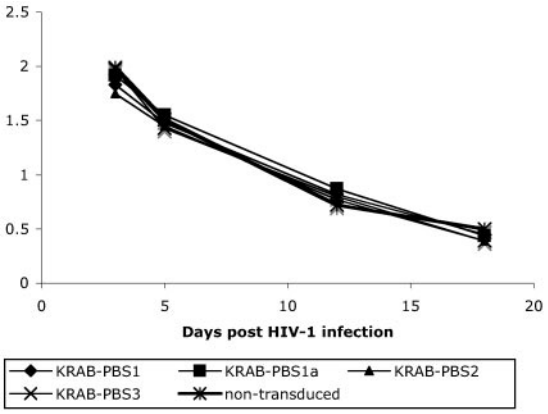

FIG. 6. Transduction of PBMCs with ZF proteins did not affect cell viability. PBMCs transduced with the indicated ZF transcription factor or nontransduced cells were challenged with HIV-1. At the indicated time points, cell viability was determined by the WST-1 assay (50).

rope and the virus used in most studies. However, the sequence of this region of the LTR varies among subtypes. Examination of the sequences from De Baar et al. (14) of the HLTR3-binding sites for a number of subtypes shows that there are few differences in the HLTR3-binding sequences, suggesting that KRAB-HLTR3 may be able to inhibit replication of these viruses (Fig. 7A). However, the F-subtype HLTR3 sequence differs greatly from the B subtype, and 9 nucleotides out of 18 differ from the B-subtype HLTR3 target sequence. In contrast, the sequence of the target site for KRAB-PBS2 is identical for the subtypes analyzed. To test the ability of KRAB-HLTR3 to regulate the F-subtype LTR, a B-subtype virus with the U3 region of the LTR replaced by the U3 sequence of an F-subtype virus was used. PBMCs were transduced with KRAB-HLTR3 and then challenged with the chimeric virus. As expected, the ability of KRAB-HLTR3 to inhibit production of the F-subtype LTR virus was severely reduced compared to the wild-type virus (Fig. 7B). KRAB-PBS2 inhibited replication of the chimeric virus and the B-subtype virus to the same degree, as expected, since both of the viruses tested contain the PBS from the B subtype. However, the KRAB-PBS2-binding site sequences are identical in the F subtype and the B subtype, so it would be expected that KRAB-PBS2 would be able to inhibit F-subtype viruses. Thus, while there are limitations in the potential use of KRAB-HLTR3 as an anti-HIV-1 therapeutic agent, KRAB-PBS2 could potentially be used to treat any known HIV-1 strain.

A major difficulty in AIDS treatment is the ability of HIV to mutate rapidly and develop resistance to therapeutic agents. We chose the primer-binding site as the target site for our transcription factors because it is conserved in all known subtypes of HIV-1, suggesting that this sequence is immutable for viral replication. Indeed, many studies have shown that mutations to the PBS cause reduced rates of virus production. Of the four ZF transcription factors we designed to bind to the PBS, KRAB-PBS2 most effectively repressed viral transcription and replication. We wished to determine whether repeated exposure of HIV-1 to KRAB-PBS2 would result in

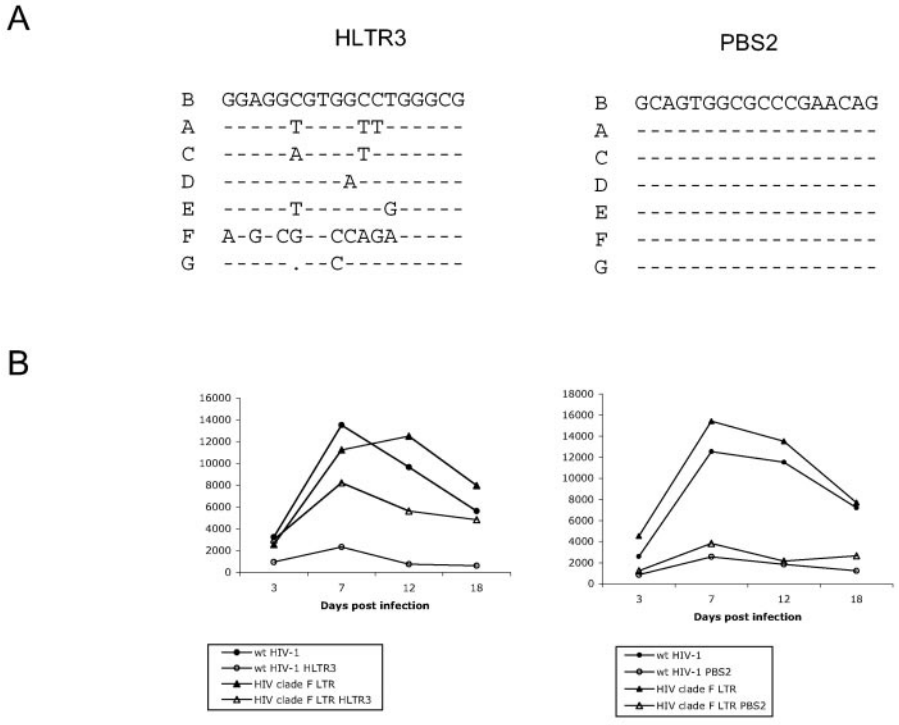

FIG. 7. Reduced effectiveness of KRAB-HLTR3 on the F-subtype LTR. (A) Sequence comparison of the HLTR3 and PBS2 ZF-binding sites in different HIV-1 M-group subtypes. Dashes indicate sequence identity, and dots indicate deletions (sequence information is taken from reference 14). (B) PBMCs transduced with either KRAB-HLTR3 (left) or KRAB-PBS2 (right) were challenged with B-subtype viruses containing either wild-type or F-subtype LTR. Virus replication was monitored by measuring p24 production.

mutations that would allow the virus to escape regulation. PBMCs transduced with KRAB-PBS2 were infected by the NL4-3 strain of HIV-1, and the virus produced was collected and was used to infect a new population of KRAB-PBS2-transduced PBMCs. After several rounds of infection, the resulting viruses were cloned and the primer-binding site regions of 20 clones were sequenced. Of the 20 clones, only 2 contained the wild-type PBS sequence; the remaining 18 consisted of three different sequences with one or two mutations in the PBS (Fig. 8A). The viral clone with the mutation that occurred in half of clones sequenced, designated Mut-3, was packaged into virus and used to infect PBMCs that had been transduced with the PBS ZFs to determine if this virus was resistant to regulation by the designed transcription factors. As with the wild-type virus, KRAB-PBS1, KRAB-PBS1a, and KRAB-PBS3 had no effect on Mut-3 virus replication. KRAB-PBS2 was able to repress Mut-3 virus replication, but much less effectively than repression of wild-type virus (Fig. 8B). In addition, the mutated virus produced much lower levels of virus than the wild-type HIV-1. These results suggest that the mutations to the PBS decrease the affinity of KRAB-PBS2 for its target sequence. To test this, an EMSA was performed to determine if the PBS2 ZF could bind to the Mut-3 sequence. We found that the PBS2 ZF bound so weakly to this sequence that the binding constant for this interaction could not be calculated (Fig. 8C). Thus, it appears that the PBS region can tolerate mutation to escape regulation by the ZF, but at the cost of efficiency of viral production.

DISCUSSION

The PBS sequence is identical in all HIV-1 subtypes identified, and all natural strains of HIV-1 use $tRNA_3^{Lys}$ for initiation of reverse transcription (31). Studies examining the effects of mutation have found that altering the sequence of the PBS results in decreased viral replication and that mutant viruses rapidly revert to the native $tRNA_3^{Lys}$ priming site (42, 46). These key features make therapies that take advantage of conserved nature of this region promising. In this study, we designed, expressed, and characterized artificial zinc finger transcription factors that regulate HIV-1 transcription through binding to the highly conserved primer-binding site. Of the four ZF transcription factors that we tested, only one, KRAB-PBS2, was able to inhibit HIV-1 viral production. Curiously, two other proteins, KRAB-PBS1 and KRAB-PBS1a, bound to the PBS sequence, as demonstrated by the ChIP assay, and inhibited the HIV-1 LTR in transient reporter assays, but they did not inhibit viral replication in PBMCs. One possible explanation is that in the assays with both the transient and stable reporters, KRAB-PBS2 was a slightly better repressor than the other two proteins. Thus, it may be that there is some threshold level of transcriptional repression that is necessary for effective inhibition of viral replication; of the ZFs tested, only KRAB-PBS2 reached this level.

KRAB-PBS3 was unable to regulate the HIV-1 LTR in any of our assays and was unable to bind to the LTR in the nucleus, as shown by the ChIP assay, despite having a K_D value for the double-stranded DNA-binding site comparable to those of the other ZFs that were tested by the gel shift assay. One possibility is that KRAB-PBS3 binds to the $tRNA_3^{Lys}$ gene, which contains the same sequence as the HIV-1 PBS. Since there are multiple copies of tRNA genes in the nucleus, it could conceivably outcompete the single integrated copy of the HIV genome for the binding of KRAB-PBS3. However, in the transient transfection of the reporter, thousands of copies of the HIV LTR were present in the nucleus, yet KRAB-PBS3 still failed to repress transcription from the LTR (Fig. 2). Also, while direct binding of KRAB-PBS3 to the $tRNA_3^{Lys}$ gene was not examined, no repression of $tRNA_3^{Lys}$ was seen by Northern blot (Fig. 3). Thus, it is possible that in a cellular environment, the LTR has a conformation that does not allow binding of KRAB-PBS3. This phenomenon was observed for other ZF transcription factors designed to bind to the HIV-1 LTR. In studies by Reynolds et al. and Kim et al. (30, 45), multiple polydactyl transcription factors were constructed that targeted the Sp1-binding sites of the LTR; in both studies, the majority of the proteins showed no regulation when tested by reporter assays. These findings demonstrate the need to use multiple assays to determine the effectiveness of designed transcription factors. Hopefully, as more ZF transcription factors are constructed and tested, the parameters for target site selection will become clearer. Recently released web-based software now allows for the automatic design of artificial zinc finger transcription factors (http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php).

To determine whether long-term exposure of HIV-1 to KRAB-PBS2 would induce mutations in the PBS that reduced the effectiveness of the repressor, we selected for escape variants of virus by passaging virus in multiple rounds using cells transduced with KRAB-PBS2. We did indeed find that the resulting virus contained mutations in the PBS and was more resistant to regulation by KRAB-PBS2; however, this virus also had delayed replication kinetics compared to the wild type. The viral mutant we tested had a CC-to-GA mutation in the sixth and seventh nucleotides of the PBS (Fig. 8A). Mutational analyses of the PBS suggest that the first six nucleotides of the HIV-1 PBS are necessary for initiation of minus-strand synthesis, whereas the last five nucleotides are important for efficient template transfer during plus-strand synthesis (9, 46, 57). A subsequent study, using a replication competent virus with a PBS complementary to $tRNA_3^{Lys}$ in nucleotides 1 to 6 and complementary to $tRNA^{Phe}$ in nucleotides 7 to 18, along with a five-nucleotide insertion downstream of the PBS, tested viruses with point mutations in each of the first six nucleotides for their ability to replicate. Of two viruses with mutations at the sixth nucleotide, the mutant with a C-to-A mutation was not infectious. The mutant with a C-to-U mutation was infectious, although the appearance of virus was delayed relative to the wild type (56). Consistent with our observations here, some mutations at the sixth nucleotide of the PBS are tolerated, as was the C-to-G mutation we observed at the sixth nucleotide.

The fact that the mutated virus appears to be viable suggests that the virus may be using an alternative tRNA to prime reverse transcription. However, a search of the database found no tRNAs that match the sequence of this new PBS. Thus, it is likely that $tRNA_3^{Lys}$is the primer and that these mutations arise during reverse transcription and are continuously selected by the pressure to escape regulation by KRAB-PBS2 instead of being repaired. In most studies of mutation of the PBS, viruses with a mutated PBS that are cultured with no selective pres-

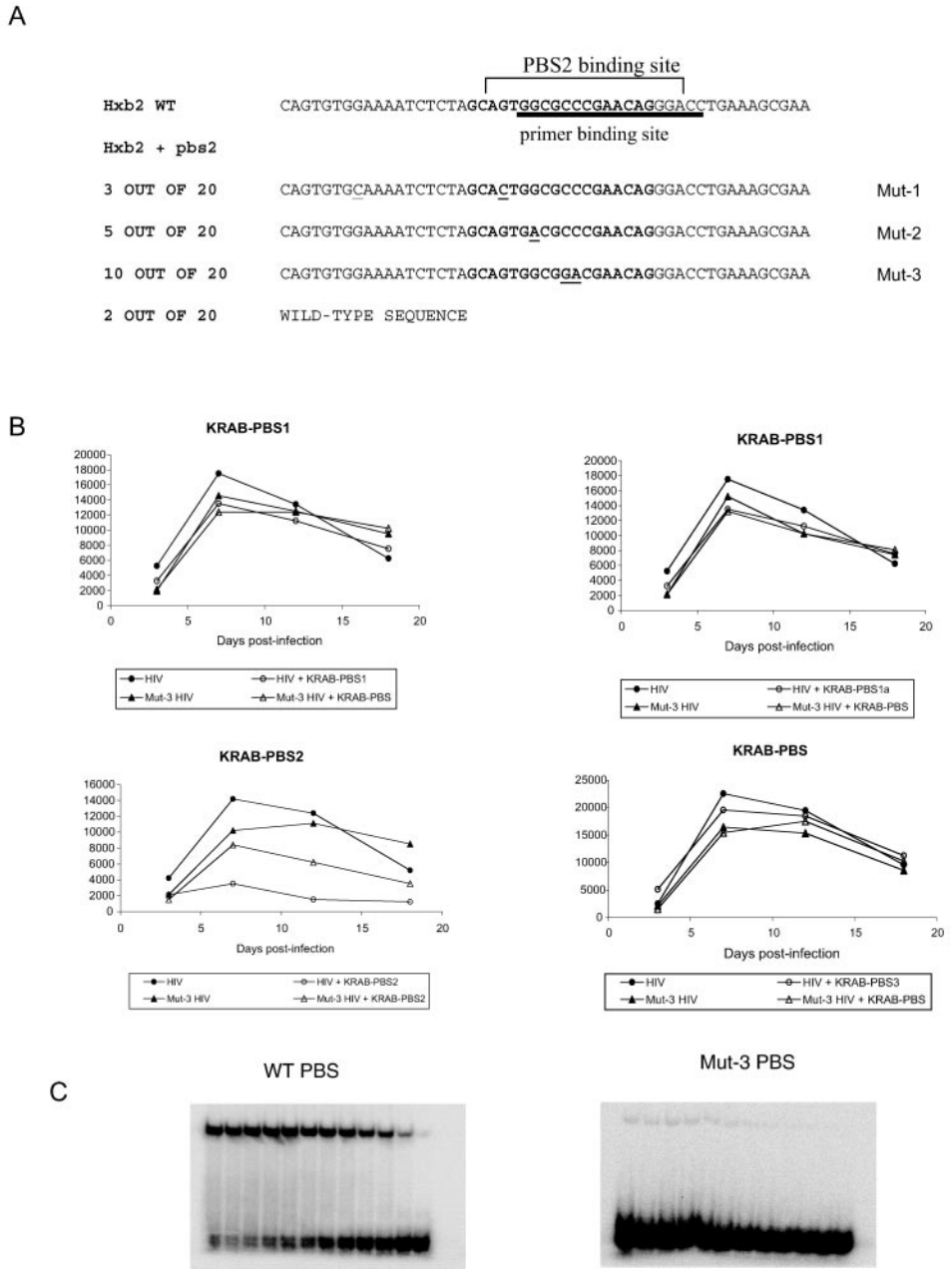

FIG. 8. Mutation of the primer-binding site in response to zinc finger repression. (A) PBMCs were transduced with KRAB-PBS2 and infected with HIV-1. Virus was collected from the supernatant and used to reinfect a new population of transduced cells. After several rounds, output virus was cloned, and the primer-binding site regions from 20 clones were sequenced. The sites of mutation are indicated by underlining. (B) Infection of KRAB-PBS-transduced PBMCs by wild-type and Mut-3 HIV. (C) Gel shift of PBS2 with wild-type and Mut-3 hairpin oligonucleotides.

sure typically revert back to the wild-type PBS sequence (11, 33, 58). However, there have been instances where mutations designed to enable HIV to use different tRNAs have resulted in stable clones. In particular, viruses have been isolated that can use $tRNA^{Lys}_{1,2}$, $tRNA^{His}$, and $tRNA^{Met}$ (1, 12, 40, 55). Importantly, these isolates have come about through laboratory manipulation of HIV-1, and there is no evidence suggesting that these mutations could occur in nature. The only natural primer variation concerns the infrequent use of $tRNA^{Lys}_{5}$, which causes a single-nucleotide polymorphism in about 5% of the HIV-simian immunodeficiency virus isolates (12, 13). These results suggest that the best strategy for using KRAB-PBS2 as a therapy for AIDS would be to discontinue treatment at intervals to allow any viruses that have mutated to revert and then resume treatment with KRAB-PBS2. During the "resting" period, alternative therapies may be used, including but not limited to ZF proteins that target other sites in the HIV-1 LTR, such as KRAB-HLTR3. However, further studies need to be performed to determine if the mutant viruses we have isolated would indeed revert to the wild-type PBS sequence in the absence of KRAB-PBS2.

RNAi has recently emerged as a potential treatment for a

number of diseases. In particular, several studies have been performed with short interfering RNA (siRNA) molecules targeting HIV-1 and have shown potent down-regulation of viral gene expression (8, 25, 26). Unfortunately, long-term studies have shown that prolonged exposure to siRNAs results in mutations of the virus that allow it to escape regulation (6, 10). Recently, a study showed that an siRNA targeting the PBS was able to inhibit HIV-1 infection (24). Although no mutation in the PBS was seen up to 14 days after exposure to the siRNA, our studies suggest that HIV can mutate the PBS region to escape targeting by RNAi. This could be a significant drawback in the use of siRNA strategies, as it has been shown that a single point mutation can be sufficient for HIV to escape regulation by siRNAs (6). However, the strategy suggested above for allowing the PBS sequence to revert may also be applied to any therapy involving RNA interference. In fact, the use of both RNA interference and transcription factors in the treatment of HIV-1 could prove to be a potent therapeutic mixture, since they act at two different levels. This approach has already been demonstrated in the regulation of the *vegf-a* gene, in which the use of artificial transcription factors and siRNA was shown to have a greater repression together than individually (32).

In conclusion, the results of this study have identified KRAB-PBS2 as a transcription factor with the potential for use in anti-HIV therapy. Like KRAB-HLTR3, KRAB-PBS2 can inhibit virus production in primary lymphocytes for extended periods with no apparent toxicity. Significantly, KRAB-PBS2 can inhibit replication of viruses that escape regulation of KRAB-HLTR3. As KRAB-PBS2 targets a sequence that is highly conserved in all known subtypes of HIV-1, it has the potential to be used as therapy in any region of the globe. As established by small-molecule approaches to HIV-1, gene-based approaches should also incorporate a cocktail of effectors to limit HIV-1 escape.

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