JVI Journals.ASM.org

Targeting IκB Proteins for HIV Latency Activation: the Role of Individual IκB and NF-κB Proteins

Guerau Fernandez,[a]* Thomas D. Zaikos,[b]* Sohrab Z. Khan,[a] Ashley M. Jacobi,[c] Mark A. Behlke,[c] Steven L. Zeichner[a,d]

Center for Cancer and Immunology Research, Children's Research Institute, Children's National Medical Center, Washington, DC, USA[a]; The George Washington University School of Medicine, Washington, DC, USA[b]; Integrated DNA Technologies, Inc., Coralville, Iowa, USA[c]; Departments of Pediatrics and Microbiology, Immunology, and Tropical Medicine, George Washington University, Washington, DC, USA[d]

Latently infected cell reservoirs represent the main barrier to HIV eradication. Combination antiretroviral therapy (cART) effectively blocks viral replication but cannot purge latent provirus. One approach to HIV eradication could include cART to block new infections plus an agent to activate latent provirus. NF-κB activation induces HIV expression, ending latency. Before activation, IκB proteins sequester NF-κB dimers in the cytoplasm. Three canonical IκBs, IκBα, IκBβ, and IκBε, exist, but the IκB proteins' role in HIV activation regulation is not fully understood. We studied the effects on HIV activation of targeting IκBs by single and pairwise small interfering RNA (siRNA) knockdown. After determining the relative abundance of the IκBs, the relative abundance of NF-κB subunits held by the IκBs, and the kinetics of IκB degradation and resynthesis following knockdown, we studied HIV activation by IκB knockdown, in comparison with those of known HIV activators, tumor necrosis factor alpha (TNF-α), tetradecanoyl phorbol acetate (TPA), and trichostatin A (TSA), in U1 monocytic and J-Lat 10.6 lymphocytic latently infected cells. We found that IκBα knockdown activated HIV in both U1 and J-Lat 10.6 cells, IκBβ knockdown did not activate HIV, and, surprisingly, IκBε knockdown produced the most HIV activation, comparable to TSA activation. Our data show that HIV reactivation can be triggered by targeting two different IκB proteins and that IκBε may be an effective target for HIV latency reactivation in T-cell and macrophage lineages. IκBε knockdown may offer attractive therapeutic advantages for HIV activation because it is not essential for mammalian growth and development and because new siRNA delivery strategies may target siRNAs to HIV latently infected cells.

Despite remarkable advances in understanding the biology of HIV, the pathogenesis of HIV disease, and the development of combination antiretroviral therapy (cART), which can lead to the restoration of immune function, vastly extend health and life, and dramatically decrease the risk of transmission, no currently available therapies can safely and effectively attack and eliminate the long-lived reservoirs of HIV latently infected cells. While other factors may play some role (1, 2), the main reason for the inability of cART to eradicate HIV infection is the existence of long-lived reservoirs of latently infected cells (3–10). One approach to attacking the reservoirs and potentially eradicating HIV involves activating HIV replication in latently infected cells while blocking new infection of cells with cART, an approach sometimes termed "shock (or "kick") and kill" (reviewed in references 4 and 11–13).

To develop new ways to activate HIV, we must understand the viral and cellular factors that influence postintegration latency and HIV activation. Much interest has recently centered on developing HIV activation therapeutics that act epigenetically (reviewed in reference 14), such as via DNA methylation and histone acetylation. Some epigenetic agents are being studied in early clinical trials, but such strategies have the potential for significant off-target effects (OTE) (15–21), and some studies have suggested that these agents may have distinct activities in different latently infected model systems (22). Other studies have worked to identify new targets (23), such as genes that can be targeted to activate HIV expression when subjected to knockdown by RNA interference (RNAi) (24–26). Multiple agents acting through different mechanisms may be needed to effectively activate all latent HIVs, or certain latent HIVs may need multiple complementary agents for activation.

HIV gene expression depends initially on long terminal repeat (LTR) activation. Starting with the initial studies of HIV gene expression, investigators considered the LTR as an activatable T cell gene (27). Expression from the LTR initially requires important basal cellular transcription factors, plus inducible factors, critically members of the nuclear factor κB (NF-κB) family, and other cellular factors (27–36).

NF-κB is an inducible transcription factor that regulates a broad range of processes (37, 38). NF-κB transcription factors are dimers produced by the combination of five different monomers (RelA [p65], NF-κB1 [p50], NF-κB2 [p52], c-Rel, and RelB). All NF-κB family members share a 300-amino-acid N-terminal Rel homology domain (RHD) responsible for dimerization, DNA binding, nuclear translocation, and interaction with IκB proteins. Only p65, c-Rel, and RelB contain a transcription activation domain (TAD), which is responsible for gene expression activation. When p50 and p52 form homodimers, they act as transcription inhibitors; p50 and p52 activate gene expression as heterodimers when combined with a TAD-containing monomer. All NF-κB monomers can form homo- or heterodimers *in vivo*, except for

Received 21 November 2012 **Accepted** 19 January 2013

Published ahead of print 30 January 2013

Address correspondence to Steven L. Zeichner, zeichner@gwu.edu.

* Present address: Guerau Fernandez, Institut de Medicina Predictiva I Personalitzada del Càncer Ctra de Can Ruti, Camí de les Escoles, Badalona, Barcelona, Spain; Thomas D. Zaikos, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, USA.

doi:10.1128/JVI.03251-12

RelB, which only can form heterodimers. There are 15 possible NF-κB dimers, but only 9 are potential transcriptional activators due to the absence in the other dimers (p50/p50, p52/p52, p50/p52, RelA/RelB, and c-Rel/RelB) of DNA binding or transcriptional activating activity (39). The different TAD-containing NF-κB subunits have distinct specificities and, presumably, functions within the cell (40, 41). The p50/p65 heterodimer is typically the most abundant activating dimer.

In HIV latency, p50 homodimers bind the κB sites in the HIV LTR and recruit histone deacetylase 1 (HDAC1), repressing expression (42). Upon stimulation, TAD-containing NF-κBs (e.g., p50/p65 heterodimers), replace p50 homodimers, activating expression (reviewed in references 5 and 43), which is also enhanced by basal and inducible transcription factors, such as Sp1 and nuclear factor of activated T cells (NFAT) (44, 45). Transcription activation leads to the production of the viral protein Tat, which binds the transactivating responsive element (TAR) in the nascent, paused HIV transcript. Tat recruits the pTEFb complex, which phosphorylates RNA polymerase II (Pol II), strongly increasing Pol II processivity and resulting in high levels of HIV gene expression.

HIV-activating approaches employing cytokines and chemokines, which act through the NF-κB axis, have been studied, including agents such as interleukin-2 (IL-2) alone and with OKT3 or gamma interferon (IFN-γ) (46–49). However, such studies showed unacceptable toxicities or ineffectiveness against latent reservoirs or both. Small molecules have also been used to activate HIV via NF-κB pathways. The best known of these is the powerful diacyl glycerol mimetic phorbol myristyl acetate (PMA; here, tetradecanoyl phorbol acetate [TPA]) (27) and its derivatives, such as prostratin (50, 51), but phorbol esters are oncogenic, and even the less toxic derivatives still show significant toxicity (50).

IκB proteins regulate NF-κB signaling. IκBs bind NF-κB dimers in the cytoplasm, preventing the NF-κB proteins from translocating to the nucleus to regulate gene expression. Five IκB proteins have been described in humans: the canonical proteins IκBα, IκBβ, and IκBε and the nonclassical proteins Bcl-3 and IκBζ. IκB members have 5 to 7 ankyrin (ANK) repeats that mediate interaction with NF-κB dimers. Some NF-κB dimers exhibit binding preferences for certain IκBs (52). Thus, the relative abundance of certain IκB proteins within the cell may affect the availability of specific NF-κB dimers for activation; activation and subsequent release of certain NF-κB dimers may differentially activate certain genes. Moreover, different cell types produce different complements of NF-κB dimers (53).

Two signaling pathways, the classical and alternative pathways, lead to nuclear translocation of NF-κB dimers. In the classical pathway, the ligand activates the NF-κB essential modulator protein (NEMO), mainly releasing RelA-, c-Rel-, and p50-containing dimers. The alternative pathway activates NF-κB-inducing kinase (NIK), only liberating p52/RelB heterodimers. Activation of the classical NF-κB signaling pathway induces phosphorylation of IκB proteins by the IκB kinase (IKK) complex, triggering its polyubiquitination and subsequent degradation by the 26S proteosome. NF-κB signaling is regulated by a negative-feedback loop where IκBα and IκBε (but not IκBβ) expression is induced by NF-κB, allowing precise modulation to counteract rapid environmental changes (54, 55). IκBβ and IκBε help damp oscillations of the IκBα–NF-κB feedback loop (56).

The IκBs have different activities and show differences in their requirement for normal cell function, growth, and development. IκBα is the best understood. NF-κB can activate IκBα expression, which serves a negative-feedback mechanism. Without IκBα, stimulation by TNF-α continues for longer times (57, 58). IκBα knockout (KO) mice have a severe phenotype (59), dying at 7 to 10 days of age with growth failure and severe dermatitis. IκBα-deficient fibroblasts still show a response to TNF-α and maintain NF-κB in the cytoplasm prior to stimulation, suggesting that the other IκBs can compensate for missing IκBα, but NF-κB nuclear localization is prolonged, suggesting that IκBα helps end the NF-κB response. IκBβ's function is less well established. However, IκBβ shows clear differences compared to IκBα in that the IκBβ promoter is not responsive to cell stimulation, and IκBβ binds p65 and c-Rel, but binds p50 less well (54, 60).

IκBε, discovered after IκBα (61, 62), has a physiologic function that is less well understood. IκBε is expressed predominantly in T cells of the thymus, spleen, and lymph nodes (63), so major sites and cell types of IκBε expression coincide with the locations of some of the main reservoirs of HIV latently infected cells. IκBε KO mice show a relatively normal phenotype, in contrast to the lethal phenotype observed with IκBα knockouts (59, 63). IκBε KO mice are identical to wild-type mice in appearance and histology and breed normally. The main differences between wild-type and IκBε KO mice are decreased $CD44^-$ $CD25^+$ T cells, and increased production of IL-1α, IL-1β, IL-1 receptor α (IL-1Rα), and IL-6 mRNAs in macrophages. The minimal phenotype of IκBε KO mice suggests that targeting IκBε in an adult animal or human would be relatively safe.

In our work, we found that IκBα and particularly IκBε small interfering RNA (siRNA) activates HIV. Targeting IκBε represents a highly plausible and likely effective new approach to activating HIV expression that may find a role in attacking the reservoir of HIV latently infected cells.

MATERIALS AND METHODS

Activating agents and siRNAs. We used tumor necrosis factor alpha (TNF-α) (10 ng/ml; Invitrogen), trichostatin A (TSA; Sigma-Aldrich) (1.5 μM), phorbol myristyl acetate PMA (here, tetradecanoyl phorbol acetate [TPA]) (20 ng/ml; Sigma-Aldrich), and combinations of the agents as positive-control treatments to activate HIV-1 from promonocytic U1 (64) and cells of the T-lymphocyte Jurkat cell-derived line J-Lat 10.6 (65). Human IκBα and IκBε siRNAs and a nonspecific siRNA pool control (D-001206-14) were purchased from Dharmacon, and validated siRNA against IκBβ was acquired from Qiagen. We designed single siRNA sequences using computational algorithms supplied by the Dharmacon, Invitrogen, Qiagen, and Whitehead websites and used siRNAs generated by Integrated DNA Technologies (IDT) (Table 1). The target sequences of the selected siRNAs (catalog numbers in parentheses) are as follows: IκBα (D-004765-02), GGACGAGAAAGATCATTGA; IκBβ (S102654953), CCCGAGGGCGAGGATGAGAAA; and IκBε (D-004766-02), GGAAGGGAAGTTTCAGTAA.

Cells and transfections. U1 latently infected cells (64) (NIH AIDS Reagent Program), an HIV latently infected cell line often used in HIV latency studies (reviewed in references 66, 67, and 68), were derived from chronically infected U937 cells, a promonocytic cell line isolated from the pleural effusion of a 2-year-old with diffuse histiocytic lymphoma. U1 cells were seeded 48 h prior to transfection at a cell concentration of 0.2×10^6 cells/ml in RPMI (Atlanta Biologicals) with 1% L-glutamine (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma), and 0.4% β-mercaptoethanol (Sigma) in 5% CO_2 at 37°C. On the day of transfection, specific siRNAs at the desired concentrations were mixed with 1.5 million U1 cells, resuspended

TABLE 1 siRNA sequences used to target IκBs

Target	siRNA no.	siRNA sequence
IκBα	1	GAGCTCCGAGACTTTCGAGGAAATA
	2	CTGGGCCAGCTGACACTAGAA
	3	AGGACGAGCTGCCCTATGA
	4	GAACATGGACTTGTATATT
	5	AAGGGTGTACTTATATCCACA
	6	GGTAGGATCAGCCCTCATT
	7	GGACGAGAAAGATCATTGA
IκBβ		CCCGAGGGCGAGGATGAGAAA
IκBε	1	ATCAACGAAGGAGTGAGTCAA
	2	GTACGACTCTGGCATTGAG
	3	GAAGCACTCACTTACATCT
	4	CTGGCTGTACATCTGGACCAA
	5	GAATTGCTGCTTCGGAATG
	6	GGAAACTGCTGCTGTGTAC
	7	CCCATGTTGGGTCAGCCTGAA
	8	GGTGTCCCATCTTATTGCT
	9	GGAAGGGAAGTTTCAGTAA
	10	GAGGCAGAGAGAAGGAAAT
	11	TTGGAGCGTCTCATCCAGTGA
	12	GAGAGAGACAGCCGTTAAA

in Nucleofector solution V (Lonza), and transfected with Amaxa program W1. The transfection efficiency, using the siGlo green transfection indicator (Thermo), for U937 was 48%. The concentration of transfected siRNAs ranged from 63 to 2,000 nM to generate a dose-response curve for each IκB siRNA. When combined, the different siRNAs targeting the three IκB genes were transfected at a concentration of 1,000 nM. TNF-α (10 ng/ml) was used as a positive control in siRNA transfections. Once transfected, U1 cells were transferred to a six-well plate that contained 1.4 ml of prewarmed 1× phosphate-buffered saline (PBS) per well. Plates were incubated for 3 h in 5% CO_2 at 37°C. Cells were transferred to a 1.5-ml microcentrifuge tube and centrifuged at 1,400 rpm for 7 min. PBS was discarded, and cell pellets were resuspended in 2 ml RPMI–10% FBS and incubated in 6-well plates for 72 h at 37°C. Twenty-four hours posttransfection, 600 μl was used to extract total RNA using the RNeasy minikit (Qiagen), and 72 h posttransfection, cells were pelleted and supernatants were stored at −80°C for further use. The relative replication capacity of the virus was determined by measuring the amount of p24 antigen produced 72 h after transfection using an enzyme-linked immunosorbent assay (ELISA) (PerkinElmer). To determine cell viability, we used an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Promega).

J-Lat 10.6 cells (65) (J-Lat full-length clone 10.6; NIH AIDS Reagent Program) were derived from the Jurkat T cell line by infection with an HIV in which *nef* sequences were replaced with green fluorescent protein (GFP) coding sequence and latently infected cells were cloned and determined to be latently infected by activation of GFP by HIV activators. Transfection efficiency, determined using siGlo, for Jurkat cells was 54%.

Real-time qRT-PCR. Total extracted RNAs (RNeasy minikit; Qiagen) from 0, 12, 24, 36, 48, 60, and 72 h posttransfection were quantified with a Nanodrop HD-1000 spectrophotometer (Thermo). Eight hundred nanograms of RNA per sample was used for cDNA preparation. The RNA was mixed with 50 μM random hexamers and 10 mM deoxynucleoside triphosphate (dNTP). The samples were incubated at 65°C for 10 min to denature the RNA and immediately chilled on ice. Eight microliters of reverse transcription-PCR (RT-PCR) mixture (1× buffer, 20 mM dithiothreitol [DTT], 200 U SuperScript II reverse transcriptase [Invitrogen], and 20 U SUPERase-In [Invitrogen]) was added to the reaction mixture, and the samples were further incubated for 10 min at 25°C for primer annealing and 50 min at 42°C and 15 min at 70°C for enzyme inactivation. Quantitative real-time RT-PCR (qRT-PCR) assays using the TaqMan system (Applied Biosystems) were used to determine the extent of knockdown of each siRNA relative to the siRNA control pool and the basal concentrations of the different IκB and NF-κB mRNAs (Table 2). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin as endogenous controls. The final volume was 50 μl (1× TaqMan gene expression assay, 1× TaqMan gene expression master mix, 50 ng cDNA). The PCR conditions were two holds of 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The detection was performed with a 7900HT Fast real-time PCR machine (Applied Biosystems).

TABLE 2 RT-PCR probe sequences

Target	Assay identification no.	Probe sequence
IκBα	Hs00153283_m1	CGGGGACTCGTTCCTGCACTTGGCC
IκBβ	Hs00182115_m1	GAAAACTACGAGGGCCACACCCCAC
IκBε	Hs00234431_m1	ACGGAGACACGCTGGTCCACCTGGC

Immunoblots. Time course experiments were performed after transfection. U1 cells were washed twice with 1× PBS and resuspended with lysis buffer. After 30 min on ice, cells were pelleted for 30 min at 13,000 rpm. Supernatants were stored at −80°C until further use. All samples were resuspended in 2× Laemmli buffer, heated at 95°C for 10 min, and normalized to cell count. Samples were fractionated on a 4 to 12% sodium dodecyl sulfate-polyacrylamide gel (NuPAGE; Invitrogen) for 2 h (Novex minicell; Invitrogen), using 1× MOPS (morpholinepropanesulfonic acid)-SDS running buffer, transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1 h at 90 V (Mini-Protean 3 cell; Bio-Rad), and blocked (1× PBS, 0.1% Tween 20, 5% skim milk) overnight at 4°C. Specific primary antibody antiserum (IκBα, sc-1643; IκBβ, sc-945; and IκBε, sc-7275) (Santa Cruz Biologicals), diluted 1/200, was used to detect the IκB proteins as well as basal protein concentration with a horseradish chemiluminescent system (ECL-Plus; PerkinElmer). The NF-κB subunit basal concentration was determined using primary antibodies to p65 (436700; Invitrogen), p50 (ab72138; Abcam), and p52 (05-361; Millipore). As a loading control, β-actin was used and detected with antibody sc-1615 (Santa Cruz). Protein quantification was performed using ImageJ software (http://rsbweb.nih.gov/ij/).

p24 assay. To determine viral induction using siRNA or the positive-control activating agents, p24 antigen was measured in U1 and J-Lat 10.6 latently infected cell supernatants using an enzyme-linked immunosorbent assay (ELISA) (Perkin-Elmer). Seventy-two hours posttransfection, cells were pelleted at 1,400 rpm for 7 min. Three hundred microliters of supernatant was stored at −80°C until p24 was determined, as described by the manufacturer.

Co-IP. For coimmunoprecipitation (co-IP), a total of 10×10^6 U1 cells were collected and washed twice in chilled 1× PBS at 1,400 rpm for 7 min. Cells were resuspended in 1 ml of lysis buffer (1% Triton X-100, 3 mM EDTA, 2 mM DTT, 1 mg/ml bovine serum albumin [BSA], 20 mM Tris-HCl [pH 7.0], 50 mM NaCl, and protease inhibitor tablet Complete Ultra Mini [Roche]) and incubated on ice for 1 h with mixing every 15 min. Samples were centrifuged at 14,000 rpm for 30 min at 4°C, and supernatants were transferred to a new microcentrifuge tube. Cell lysates were precleared with True Blot anti-rabbit immunoprecipitation (IP) beads (Thermo Scientific) for 15 min at 4°C. We incubated 400 μl of postclear supernatant (4×10^6 cells/reaction) overnight at 4°C with the specific IκB antibodies or isotype controls (5 μg/reaction). After incubation with the primary antisera, the samples were incubated for 4 h at 4°C with True Blot anti-rabbit IP beads and washed twice with lysis buffer and two more times with wash buffer (1/10 lysis buffer). Supernatants were discarded, and beads were frozen and stored at −80°C for further use. All samples were resuspended in 2× Laemmli buffer, heated at 95°C for 10

min, and normalized to cell count. Samples were processed as described above for immunoblot analysis. Specific primary antibodies to IκBα (sc-1643), IκBβ (sc-945), and IκBε (sc-7275) (Santa Cruz), were used for the immunoprecipitation. For the NF-κB immunoblots, the following mouse monoclonal antibodies were used: p65, 436700 (Invitrogen); p50, ab72138 (Abcam); and p52, 05-361 (Millipore).

Statistical analysis. To determine the different IκB protein half-lives ($t_{1/2}$), we calculated the first-order decay using a linear regression, $t_{1/2} = \log_2/k$, with k, the slope of the linear regression, as the decay constant. To determine the correlation between p24 fold increase and mRNA knockdown for the different siRNAs targeting IκBα and IκBε, Spearman's ρ, was generated using the statistical package R (http://www.R-project.org). STATA11 (StataCorp) was used to generate Gompertz 4 component nonlinear logistic regression plots.

RESULTS

Characterization of the IκB and NF-κB proteins in U1 latently infected cells. Since our ultimate goal was to assess the role of individual IκB and NF-κB proteins in HIV activation and, in particular, to determine whether targeting IκB proteins would activate HIV, to better understand how the different components of the NF-κB pathway control HIV expression in U1 cells, we quantified the relative abundance of mRNA and proteins of the different IκBs (Fig. 1A) and NF-κBs (Fig. 1B). Protein and RNA values for the IκBs were normalized to what we determined to be the least abundant IκB, IκBε; protein values for the NF-κB subunits were normalized to what we determined to be the least abundant subunit at the RNA level, RelB. We found that the most abundant IκB species was IκBβ, with RNA present at a 21.4-fold-greater amount than that of IκBε and protein present at a 7.6-fold-greater amount than that of IκBε. IκBα RNA was present at an 8.1-fold-greater amount than IκBε, and protein was present at a 6.5-fold-greater amount than IκBε. The least abundant NF-κB subunit was RelB, followed by RelC, with RNA present at a 1.01-fold-higher level than RelB and protein at a 0.91-fold-lower level than RelB. p52 had an RNA level 146.1-fold higher than RelB and a protein level 92-fold higher than RelB. p50 had an RNA level that was 220.4-fold higher than RelB and a protein level that was 438-fold higher than RelB. p65 had an RNA level that was 883-fold higher than RelB and a protein level that was 540-fold higher than RelB. The very low relative levels of protein and RNA in these cells suggest that RelB and c-Rel play a limited part in NF-κB-mediated HIV activation.

After making estimates of the relative abundance of the different IκBs and NF-κBs, we next determined how the individual NF-κB subunits interact with the different IκBs using a qualitative coimmunoprecipitation (co-IP) assay (Fig. 1C). We found that IκBα strongly interacted with p65, p52, and p50, while IκBβ and IκBε interacted strongly with p65 and interacted less strongly with p52 and p50. If different NF-κB subunits have significantly different effects on one or another biological process, then since IκBs hold different populations of NF-κB subunits, releasing the NF-κB subunits from particular IκBs—say by specifically targeting a particular IκB for degradation—might be likely to have specific biological effects.

One of the key determinants of how activation through the NF-κB pathway initiates HIV replication must involve the kinetics of IκB destruction and replenishment following exposure of the host cell to an activating stimulus. To determine how the different IκB proteins respond when the NF-κB pathway is activated, we treated cells with TNF-α and assayed at serial times for IκB protein abundance using immunoblots (Fig. 1D). The three canonical IκB proteins showed substantially different decay and replenishment kinetics, as expected. The results showed that IκBα showed a steep decline, becoming undetectable by 5 min following TNF-α treatment. IκBβ and IκBε showed a more moderate reduction. IκBα recovered faster than IκBβ and IκBε. By 90 min after TNF-α treatment, IκBα had increased to more than double its initial amount. Both IκBβ and IκBε showed similar kinetics after TNF-α treatment, both showing a much smaller reduction in abundance than IκBα, reaching a nadir at 15 min and then increasing slowly in abundance, but not returning to the initial amounts by 120 min. These results indicate that IκBα has a much faster feedback mechanism than IκBβ or IκBε. These differences in kinetics might result from physiologically different roles of the three IκBs. IκBβ and IκBε recover more slowly than IκBα, so the effects resulting from their destruction would last longer. The co-IP results show that the pool of NF-κB dimers binding to the three IκBs is different, reinforcing the model that each IκB has a specific role in holding distinct populations of NF-κB subunits inactive in the cytoplasm or releasing distinct subunits following activation. Physiologically, IκBα may therefore play a more important role in short-term changes, while IκBβ and IκBε may influence more long-term processes in these cells.

Knockdown kinetics of IκB proteins following siRNA treatment. Treatment with the powerful inducer TNF-α strongly affected all three canonical IκB proteins, as shown in Fig. 1D. To assess the role that the different IκB proteins and their bound NF-κB subunits play in HIV activation, we knocked down the different IκB mRNAs with siRNAs specific for each IκB. In a preliminary experiment, we used a pool of four siRNAs against IκBα and IκBε purchased from Dharmacon and a single siRNA targeting IκBβ purchased from Qiagen. The IκBβ siRNA knockdown reached a level of 78%, while IκBα and IκBε knockdowns reached levels of 49% and 35% (not shown). To increase the knockdown effect of IκBα and IκBε, we assayed for knockdown produced by the individual siRNAs included in the pool and generated new ones using computational algorithms available on the Invitrogen, Qiagen, and Whitehead websites. For IκBα, we tested three more siRNAs and tested eight more for IκBε, screening a total of seven siRNAs for IκBα and 12 siRNAs for IκBε (Table 1 and Fig. 2A and B). We determined mRNA knockdown in U1 cells at 24 h posttransfection using qRT-PCR. The siRNAs that achieved the best knockdown were no. 7 for IκBα and no. 9 for IκBε. We used those siRNAs for subsequent experiments. Both siIκBα and siIκBε siRNAs targeted the 3′ (UTR) region of the specific mRNAs, while the siRNA against IκBβ was directed to the coding region (Fig. 3A). None of the siRNAs had cytotoxic effects: there was no decrease in cell viability following and IκB siRNA transfection over 72 h (Fig. 3B). We determined the knockdown kinetics of the IκB mRNAs using qRT-PCR (Fig. 3C). mRNA knockdown peaked at 12 h posttransfection and slowly recovered, returning to about 70% of baseline mRNA abundance by 72 h. We calculated the half-lives of the different IκB proteins (Fig. 3D) based upon abundances determined by immunoblotting, which also enabled us to establish on- and off-target effects for the different IκB siRNAs (Fig. 3E). The protein kinetics, determined by immunoblotting, showed patterns that differ from the kinetics observed for IκB RNA (Fig. 3E). IκBα siRNA produced a rapid decay in IκBα protein levels, with levels reaching less than 20% of their initial abundance by 12 h. IκBβ siRNA decreased IκBβ protein to levels that

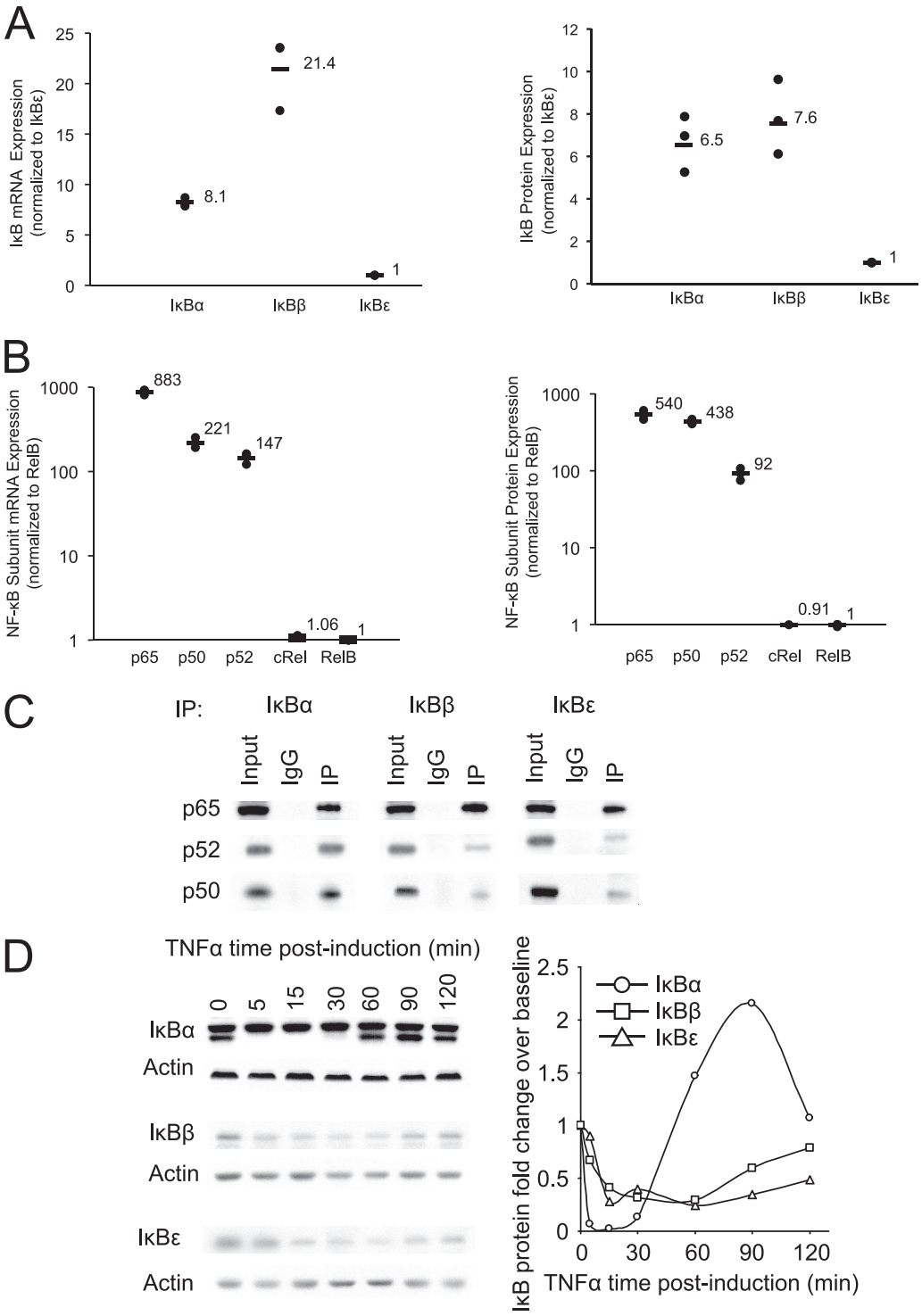

FIG 1 Regulation of the NF-κB pathway by IκB proteins in U1 HIV latently infected cells. (A) IκB mRNA quantification by RT-PCR (left panel) and IκB protein quantitation by immunoblotting (right panel). In this figure, the RNA and protein values were normalized to the levels of IκBε. The dots indicate the different replicates, and the lines represent the mean values. (B) NF-κB mRNA quantitation by RT-PCR (left panel) and NF-κB protein monomer quantitation by immunoblotting (right panel). In this figure, the RNA and protein values were normalized to the levels of RelB. (C) Coimmunoprecipitation of the three canonical IκB proteins with p65, p52, and p50 NF-κB monomers. IκBα, IκBβ, and IκBε were immunoprecipitated with specific monoclonal antibodies, and the NF-κB proteins that coprecipitated with the IκB proteins were detected by immunoblotting using monoclonal antibodies against p65, p52, and p50, as listed in the figure. The positive-input control extract was electrophoresed using 10% of the extract subject to immunoprecipitation. The negative IgG control was rabbit IgG. IκBα interacted strongly with all NF-κB subunits and IκB proteins. IκBβ and IκBε interacted strongly with p65, but interacted less strongly with p52 and p50 compared to IκBα. (D) Degradation kinetics of the different canonical IκBs. Cells were treated with TNF-α (10 ng/ml), and protein was isolated after 0, 5, 15, 30, 60, 90, and 120 min. IκB proteins and an endogenous β-actin control protein were detected by SDS-PAGE and immunoblotting (left panel). The immunoblots were scanned and quantitated densitometrically, normalized to the endogenous β-actin control, and plotted (right panel). IκBα protein decreased rapidly to undetectable levels and stayed low for approximately 30 min, after which it increased until peaking at 90 min, at about twice the baseline level. IκBβ and IκBε had slower decay kinetics, reaching nadirs at about 15 min, but never becoming undetectable. After 60 min, both IκBβ and IκBε proteins exhibited a sustained increase, although none reached baseline levels by 120 min.

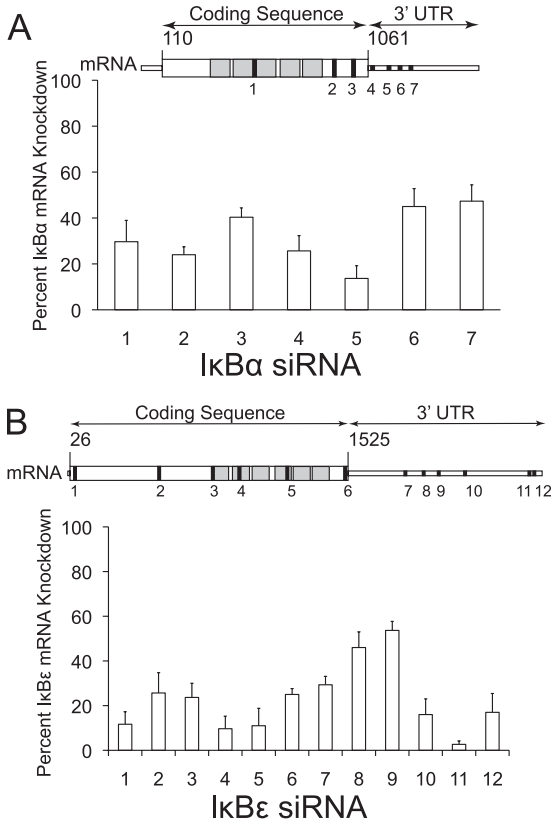

FIG 2 Identification of highly active IκBα and IκBε siRNAs. (A) Determining the knockdown activity of seven siRNAs targeting IκBα mRNA by qRT-PCR. The location of the siRNA binding sites in the IκBα mRNA is indicated in the schematic diagram at the top of the figure. Gray regions within the coding sequence (flanked by the nucleotide residues) of both mRNAs represent ANK repeats. IκBα siRNA 7 had the best knockdown effect in U1 latently infected cells. It was used in subsequent experiments. (B) Quantification of knockdown by qRT-PCR of 12 siRNAs targeting IκBε mRNA. The location of the siRNA binding sites in the IκBε mRNA is indicated in the schematic diagram at the top of the figure. Gray regions within the coding sequence (flanked by the nucleotide residues) of both mRNAs represent ANK repeats. IκBε siRNA 9 had the best knockdown effect in U1 latently infected cells and was used in subsequent experiments. Results are the means ± standard deviations (SD) from three independent experiments.

were also about 20% of baseline, but for IκBβ, the nadir was achieved after 48 h. IκBε siRNA also achieved a nadir at about 48 h, but the IκBε siRNA did not reduce IκBε protein levels to the extent IκBα, nor did IκBβ siRNA. None of the siRNAs had significant off-target effects at the protein level: that is, each IκB siRNA affected only the RNA and protein levels of the targeted IκB, with only minimal effects on the other IκBs, making it unlikely that the activation observed would be due to the knockdown of a nontargeted IκB. The data sets made it possible to estimate the half-lives of the different IκBs in the U1 cells (Fig. 3D). We found that IκBα had the shortest half-life, 5 h, while we found that IκBβ had a half-life of 17 h and IκBε had a half-life of 33 h. Thus, while the knockdown of IκBε was not as quantitatively large as knockdown of IκBα, IκBε knockdown persisted much longer. These results demonstrate that our siRNAs can specifically knock down individual IκB proteins and that IκB protein decay and replenishment kinetics following siRNA knockdown are comparable to those observed following treatment with TNF-α (Fig. 1C). This suggests that the IκB siRNA knockdown produced changes in IκB protein levels that, while not as quantitatively extensive as those seen with TNF-α, nevertheless shared the basic kinetic characteristics seen with TNF-α treatment. The longer half-lives and slower replenishment kinetics of the IκBβ and IκBε proteins reinforced the hypothesis that there might be significant differences in the biological effects of knocking down the different IκB proteins, suggested by the finding that the different IκBs hold different populations of NF-κB subunits. In addition to the release of different populations of NF-κB subunits, the differences in the kinetics of knocking down the individual IκB proteins further suggest that targeting individual IκB proteins may have different biological effects. The specificity observed in knocking down the individual IκB proteins indicates that the specific IκB siRNAs could be used to dissect out the different roles that the IκB proteins and the NF-κB subunits bound to those IκBs play in biological processes, including HIV activation.

IκBα and IκBε knockdown induction of HIV activation. Having established that our siRNAs could knock down individual IκBs at the RNA and protein levels and having characterized the performance characteristics of those siRNAs, we could then use those reagents to study the involvement of individual IκBs and the NF-κB subunits held by those IκBs in the maintenance of HIV latency and HIV reactivation. We performed a series of transfections to determine the activation dose response with specific IκB siRNAs (Fig. 4A). For each concentration, we determined the extent of mRNA knockdown as well as the increase in cell supernatant HIV p24 antigen, determining the fold increase seen with the transfection, normalized to the siRNA control. There appeared to be a clear dose response associating HIV activation with the extent of the IκB knockdown for both IκBα and IκBε siRNAs (Fig. 4B). Although IκBβ siRNA achieved the greatest knockdown, reaching almost 75% of baseline, IκBβ siRNA produced no viral reactivation. However, IκBα and IκBε siRNAs both reactivated HIV from latency. With IκBα siRNA, knockdown reached levels of ~60%, which produced substantial activation, with p24 levels increasing by >5-fold. Surprisingly, the IκBε siRNA had a larger effect. With the IκBε siRNA, we were able to achieve knockdown levels of about 75%, which produced activation of more than a 10-fold increase in HIV p24 antigen in the cell supernatant. At equivalent achieved levels of knockdown (up to 60%), IκBε siRNA appeared to be a more potent activator, which was unexpected given the relatively minor role that IκBε had been thought to play in the regulation of NF-κB-controlled gene expression and the relatively smaller amounts of IκBε present in the cell. A threshold effect was also apparent for IκBε knockdown, with a large increase in HIV activation when IκBε knockdown exceeded about the 50% level.

To show that knocking down IκBα or IκBε specifically produced HIV reactivation, and to explore whether some nonspecific effect of the siRNAs studied in the experiments of Fig. 3 and 4 might have led to HIV activation, we determined the HIV reactivation capabilities of the different IκBα and IκBε siRNAs we initially tested for IκB mRNA knockdown ability (Fig. 2). These two sets of siRNAs targeted diverse regions of the mRNAs and exhibited a spectrum of both IκB knockdown activity and, when tested for activation, HIV activation ability (Fig. 5). We observed a clear correlation by Spearman's ρ (IκBα, $\rho = 0.96$; and IκBε, $\rho = 0.93$) between the ability of the siRNAs to knock down IκB mRNA and activate HIV (Fig. 5B and D), although the two IκBε siRNAs that were most effective at knocking down IκBε mRNA had a some-

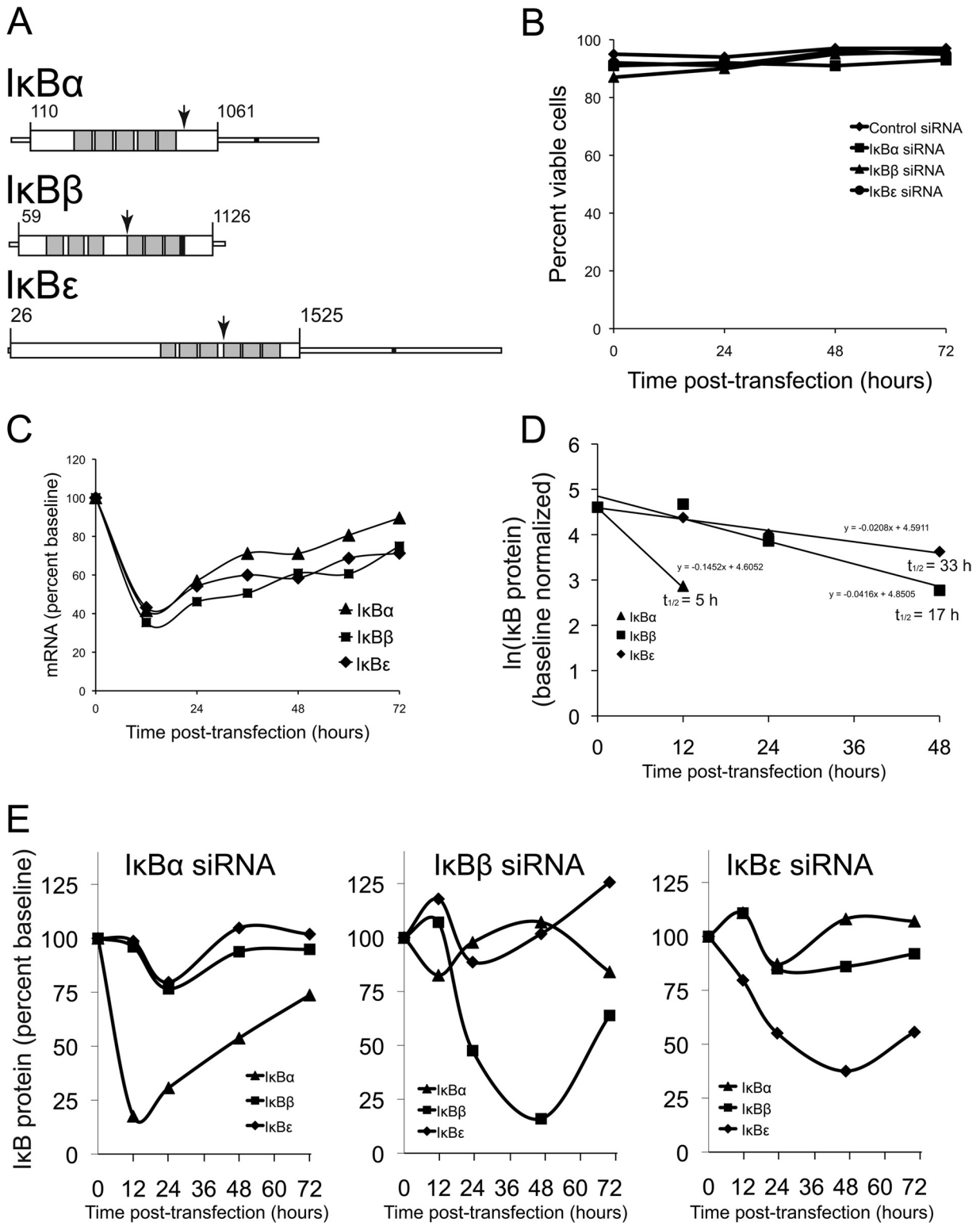

FIG 3 Knockdown performance characteristics of siRNAs targeting IκBs. (A) Diagram of the three IκB mRNAs showing regions targeted by the siRNAs (black) and RT-PCR probes used (arrows). (B) Cell viability following transfection with IκB siRNAs, determined by MTS assay. Little to no decrease in cell viability was observed over 72 h. (C) IκB mRNA levels after siRNA transfection. Individual IκB mRNAs were quantitated by qRT-PCR at serial times following transfection with IκB siRNAs and normalized to endogenous GAPDH. All mRNAs were knocked down substantially, with a nadir for each IκB mRNA at 12 h. (D) The half-life for each protein was calculated determining the slope of each protein knockdown. IκBα had a short half-life (5 h), compared to IκBβ (17 h) and IκBε (33 h). (E) IκB protein levels after siRNA transfection. Individual IκB proteins were quantitated by immunoblotting using specific monoclonal antibodies and densitometry at serial times following transfection with IκB siRNAs and normalized to β-actin. Each subpanel lists the specific siRNA at the top of the subpanel. In contrast to the results for the IκB mRNA levels, the IκB protein levels reached nadirs at different times following transfection, exhibiting substantially different half-lives and different recovery kinetics. IκBα protein reached a nadir at 12 h, similar to that seen for IκBα mRNA, and started to recover back to baseline relatively quickly. In contrast, IκBβ and IκBε proteins did not reach a nadir until 48 h after transfection and then recovered slowly. These results are consistent with those seen following treatment with TNF-α (Fig. 1). The individual siRNAs exhibited little off-target effect for the other nontargeted IκBs. The half-life for each protein was calculated determining the slope of each protein knockdown (D).

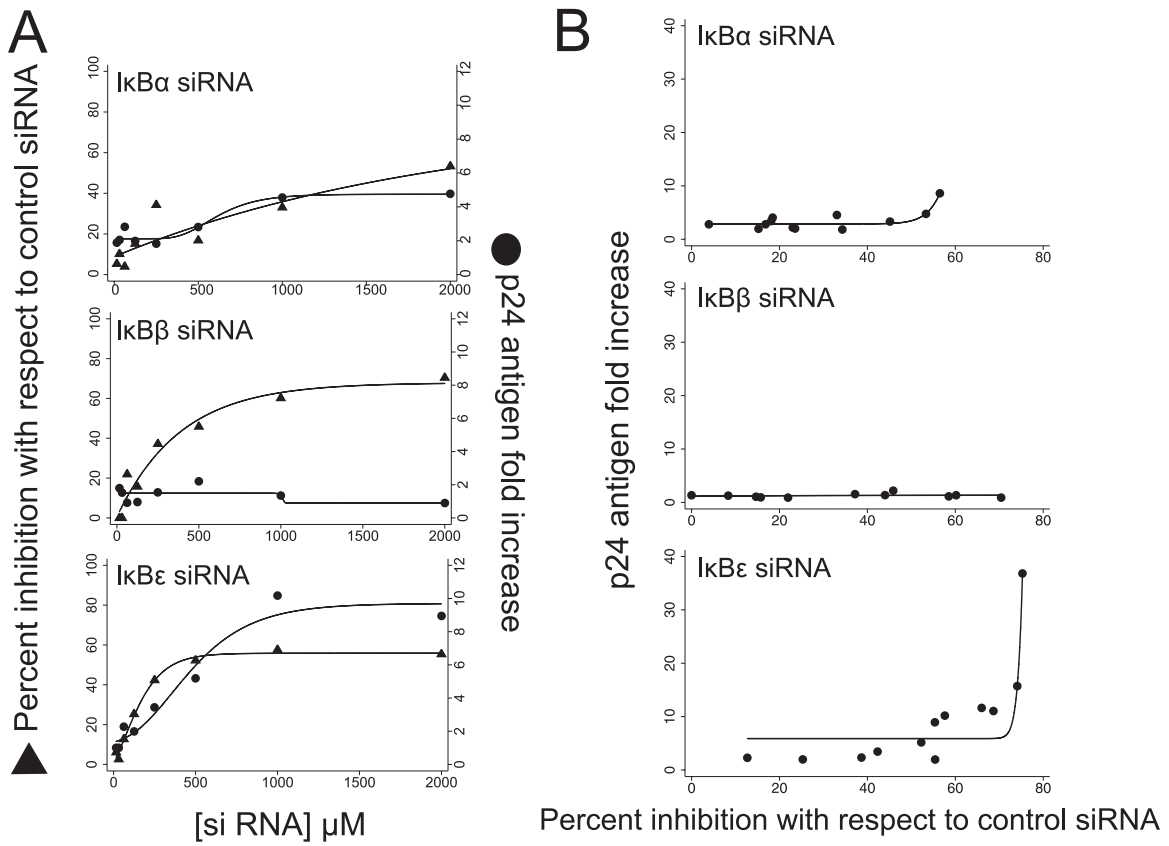

FIG 4 IκB protein knockdown and HIV activation dose responses of IκB siRNAs. (A) Individual siRNAs targeting IκBα (upper panel), IκBβ (middle panel), and IκBε (lower panel) were transfected into U1 cells. Expression of the IκB mRNAs was determined by qRT-PCR after 24 h (solid triangles), and the effect of the siRNAs on HIV activation was determined by enzyme-linked immunosorbent assay (ELISA) for HIV-1 p24 viral antigen at 72 h posttransfection (solid circles). IκBα and IκBε induced HIV reactivation. IκBβ knockdown had no effect on HIV activation. Each graph is representative of three independent experiments. (The curves are Gompertz 4 component nonlinear logistic regression plots [STATA11].) (B) The relationship between knockdown of the different IκB mRNAs, determined by qRT-PCR and viral activation, quantified by p24 antigen, was examined using different concentrations for IκBα (upper panel), IκBβ (middle panel), and IκBε (lower panel) siRNAs. Results are pooled from three independent experiments. Activation was quantified as the fold increase in p24 antigen over baseline. The extent of IκB mRNA knockdown was quantified with respect to the levels observed following transfection of the control siRNA. Knocking down IκBβ expression had no effect on HIV activation, while knocking down IκBα had a moderate HIV-activating effect. IκBε knockdown strongly activated HIV-1 expression, with a >15-fold increase in p24 production. The data also suggest that there is a strong threshold effect: when IκBε knockdown exceeds ~55%, HIV activation increases sharply. (Curves are Gompertz 4 component logistic regression plots [STATA11].) R^2 values were as follows: IκBα, 0.80; IκBβ, 0.02; and IκBε, 0.85.

what disproportional effect on HIV activation, reinforcing the findings of the dose-response study (Fig. 4B), in which we observed a threshold effect for increased HIV activation. The findings that many different siRNAs targeting many different regions of the mRNA were effective in both knocking down mRNA and activating HIV and that the HIV-activating activity correlated with the IκBε knockdown activity argue that the HIV activation was caused by the IκB mRNA knockdown and not primarily by some other nonspecific or off-target effects of the siRNAs.

Effects of combined knockdown of IκBα and IκBε on HIV activation. After establishing that both IκBα and IκBε siRNAs effectively activate HIV in latently infected cells, we wanted to determine whether the IκBα and IκBε siRNAs would have additive effects when transfected together and to compare the effects of the IκBα and IκBε siRNAs with those of conventional HIV activators. We also wanted to confirm that the HIV-activating ability of the IκB siRNAs was not limited to a single HIV latently infected cell line or to only monocytic cells. We therefore transfected the IκBα and IκBε siRNAs alone and in pairwise combinations into both the U1 monocytic and Jurkat-derived J-Lat 10.6 lymphocytic cell lines and also treated the cells with positive-control activators, TNF-α, TSA, and TPA, which activate HIV through both the NF-κB pathway and by other mechanisms (Fig. 6). TNF-α produced the greatest activation (325- and 80-fold increases in U1 and J-Lat 10.6 cells, respectively) followed by TPA (100- and 30-fold increases) and TSA (10- and 2-fold increases). The experiments showed that IκBα and IκBε activate HIV in both the monocytic U1 cells, as seen in the previous figures and in the lymphocytic J-Lat 10.6 cells. While the HIV-activating abilities of the IκBα and IκBε siRNAs were substantially less than those of the well-known highly effective (and toxic) HIV activators TNF-α, and TPA, the IκBα and IκBε siRNAs activated HIV about as well as the histone deacetylase inhibitor TSA, a representative of a class of compounds that has attracted wide interest as potential agents to attack and purge the reservoir of HIV latently infected cells. The results confirmed that IκBβ siRNA had no effect on viral reactivation in either cell line when transfected into the cells alone and did not add to the activating effect of either IκBα or IκBε siRNAs

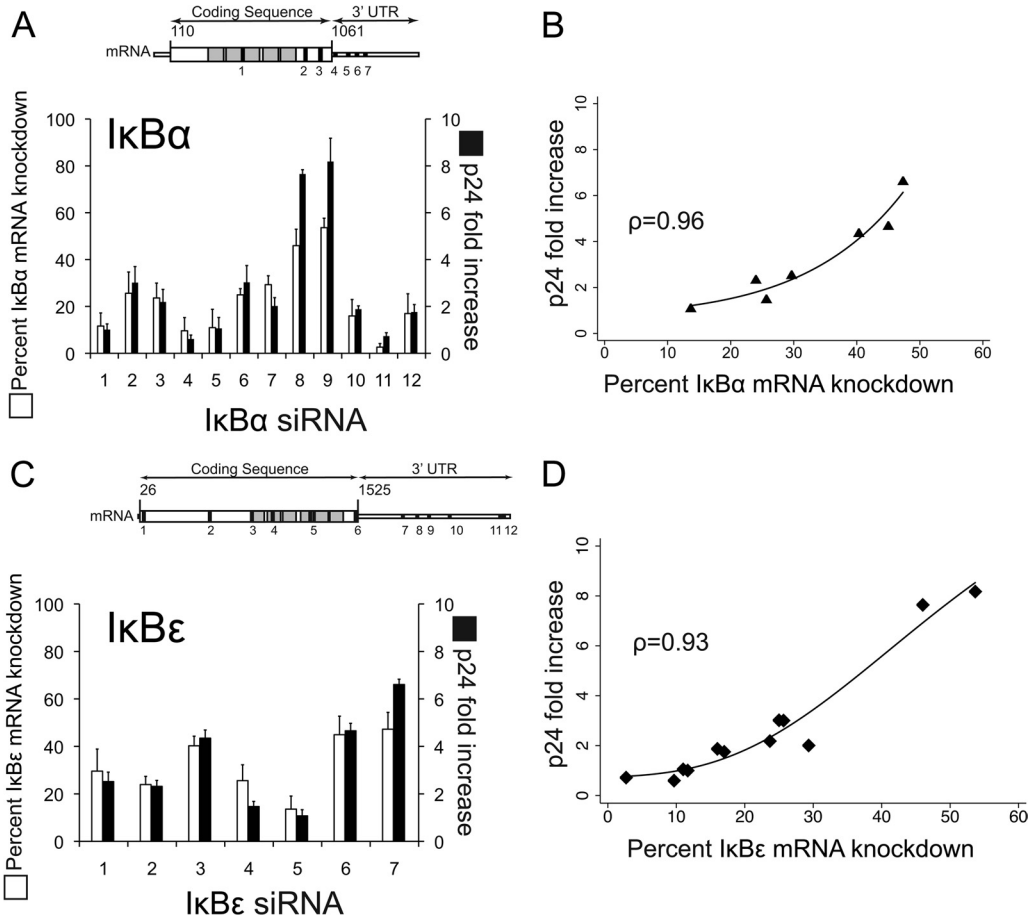

FIG 5 Viral activation using different IκBα and IκBε siRNAs. (A) Effects of different IκBα siRNAs on IκBα knockdown and HIV activation. The locations on the mRNA targeted by seven different IκBα siRNAs are shown in the schematic at the top of the panel. The bar graph shows simultaneously the knockdown effect (white bars) (Fig. 2) of the siRNAs, assayed by qRT-PCR, and the fold increase in HIV p24 antigen produced by each siRNA assessed using a p24 ELISA (black bars). (B) The panel shows the p24 fold increase as a function of IκBα knockdown for the different siRNAs. Spearman's correlation was calculated ($\rho = 0.96$). (C) Effects of different IκBε siRNAs on IκBε knockdown and HIV activation. The locations on the mRNA targeted by 12 different IκBε siRNAs are shown in the schematic at the top of the panel. The bar graph shows simultaneously the knockdown effect (white bars) (Fig. 2) of the siRNAs, assayed by qRT-PCR, and the fold increase in HIV p24 antigen produced by each siRNA assessed using a p24 ELISA (black bars). (D) The panel shows the p24 fold increase as a function of IκBε knockdown for the different siRNAs. Spearman's correlation was calculated ($\rho = 0.93$). For both IκBα and IκBε siRNAs, the extent of HIV activation correlated with the extent of IκB knockdown. In the schematic diagrams, gray regions within the coding sequence (flanked by the nucleotide residues) of both mRNAs represent ANK repeats. Results are means ± SD from three independent experiments.

when transfected into the cells in combination with those siRNAs. Interestingly, IκBα and IκBε siRNAs showed additive effects when both IκB siRNAs were transfected into both cell lines.

DISCUSSION

NF-κB was recognized very early on as a key activator of HIV expression, so when interest began to build in finding ways to activate HIV as a component of a strategy to deplete the reservoir of HIV latently infected cells, much research activity initially focused on HIV activation via NF-κB pathways. However, recognition that the available NF-κB-directed activators were toxic and relatively nonspecific, together with an increasing appreciation for the importance of other levels of HIV expression control, such as epigenetic modifications, led to a shift away from studies of the NF-κB pathway as a likely effective approach for HIV activation and reservoir depletion. Our finding that IκBε siRNA can activate HIV suggests not only that IκBε might be a reasonably specific and effective target for HIV activation, but also more broadly that specificity and selectivity for HIV activation may yet be achieved by selectively targeting specific elements of NF-κB pathways. While our IκBε siRNAs activated HIV reasonably well, particularly compared to some of the activation strategies that are currently the subject of considerable research, like HDAC inhibitors (Fig. 6), it would certainly be better if IκBε siRNAs activated HIV even more effectively. Since we observed transfection efficiencies of only ~50%, employing more effective delivery methods should substantially increase HIV activation by IκBε siRNAs. However, effective HIV activation even at relatively low levels of transfection efficiency also suggests that IκBε knockdown might, in fact, prove to be a reasonable clinical strategy, since *in vivo* clinical siRNA delivery can be challenging.

Our finding that IκBε siRNA can activate HIV was unexpected. IκBε was thought to function mostly to modulate and dampen potentially extreme autoregulatory feedback loops created by IκBα's strong response to NF-κB (56). The fact that IκBε comprises a small minority of the IκBs within a cell initially suggested

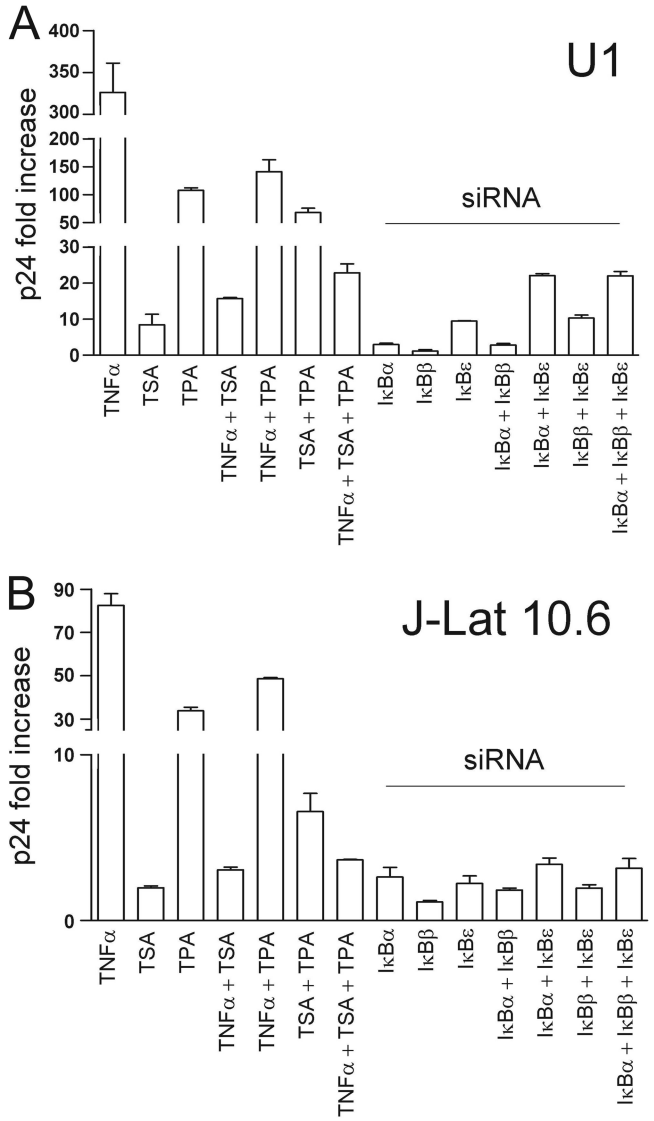

FIG 6 Effects of IκBα, IκBβ, and IκBε siRNAs in pairwise combinations and in comparison with other latency activators, TNF-α, TPA, and TSA, in U1 and J-Lat 10.6 HIV latently infected cells. (A) Promonocytic U1 latently infected cells. (B) T-lymphocyte Jurkat-derived J-Lat 10.6 latently infected cells. The IκB siRNAs were transfected into the cells singly and in pairwise combinations. The cells were also treated with the conventional HIV-activating agents TNF-α, TPA, and TSA. In the U1 cells, IκBε siRNA produced a large increase in HIV p24 antigen (~10-fold increase), comparable to that seen with TSA alone. Knocking down IκBα in addition to IκBε had an additive effect. The combination of IκBα and IκBε siRNAs produced an ~20-fold increase, similar to the values obtained with TNF-α plus TSA or TNF-α plus TSA plus TPA. In the J-Lat 10.6 cells, IκBα or IκBε siRNAs produced an ~2.5-fold increase, comparable to that with TSA alone, which increased when the siRNAs were combined. The combination of IκBα and IκBε siRNAs produced an ~3.5-fold increase, similar to the values obtained with TNF-α plus TSA or TNF-α plus TSA plus TPA. IκBβ siRNA had no effect on HIV activation, as expected. Results are the means ± SD from three independent experiments.

that targeting IκBε alone would not have much of any effect at all, an idea reinforced by the observation that IκBε KO mice have a minimal phenotype (63), in contrast to the lethal phenotype observed with IκBα KO mice (59).

Why would targeting IκBε have such a disproportionate effect on HIV activation? Our data suggest a few possibilities. First, the relatively low abundance of IκBε RNA and protein may mean that given amounts of siRNA can lead to a greater fractional knockdown of IκBε, a hypothesis supported by the observation that there appears to be a threshold effect in IκBε knockdown, with greater increases in HIV activation observed with knockdown levels beyond 50%. Second, the kinetics of IκBε destruction and replenishment may make IκBε knockdown more effective at activating HIV. Turning on the "lytic switch" for HIV replication requires essentially two hits: an increase in initial basal transcription ("hit 1"), which must be sustained long enough for some Tat to be translated in the cytoplasm and then transported back to the nucleus to bind TAR on the nascent HIV transcript ("hit 2"), which results in the massive increase in expression that initiates lytic replication. The prolonged IκBε kinetics that we observed may favor a relatively prolonged activation of basal HIV expression produced by the NF-κB subunits released by IκBε, an activation prolonged sufficiently to switch HIV onto the lytic replication pathway. This kinetic argument may also help explain the synergy observed when cells were transfected with IκBα plus IκBε siRNA. Since we observed that IκBα abundance decreased early, while IκBε abundance decreased later after siRNA transfection, the combination of both siRNAs should produce a more prolonged stimulus of the HIV LTR. Third, some of the effect may be due to the specific NF-κB subunits released from IκBε. We found that IκBε holds proportionately more p65 than p52 or p50, compared to IκBα. The inhibitory NF-κB dimer bound to the quiescent HIV LTR is a p50 homodimer (69). The principal activating NF-κB dimer for the LTR is a p50/p65 heterodimer. If knocking down IκBε predominantly releases p65 for transit to the nucleus, then the p65 released from IκBε may convert the inhibitory p50 homodimers into activating p50/p65 heterodimers. While these explanations are plausible, a definitive determination of the explanation will have to await further experiments.

We found that both IκBε and IκBβ hold disproportionately more p65 and p50, and our siRNAs were at least as effective at knocking down IκBβ, when normalized to the baseline amount of IκB, as they were at knocking down IκBε: so why then didn't the IκBβ siRNA also activate HIV? One possible explanation lies in the relative abundance of the different IκBs. IκBβ was more than 20-fold more abundant than IκBε. If there is some absolute threshold effect—some threshold below which the IκB has to be knocked down to activate HIV—then it may simply not be possible for the siRNA to knock down the IκBβ below that level. In addition, IκBβ traffics between the nucleus and cytoplasm differently from other IκBs, or at least differently from IκBα (69, 70; reviewed in reference 71). IκBα has a nuclear export signal (NES), while IκBβ does not, and unlike IκBα, IκBβ can mask the nuclear localization signal (NLS) of bound NF-κB (69), so IκBβ does not shuttle between nucleus and cytoplasm as IκBα does (70), and IκBβ, once degraded, is first resynthesized as a hypophosphorylated form (60, 72). If one of the mechanisms for HIV activation by IκBα and IκBε siRNAs, but not IκBβ siRNA, is the conversion of inhibitory p50 homodimers by relatively larger amounts of p65 released by the IκBs, the lack of shuttling by IκBβ could also help account for the lack of activation observed with IκBβ siRNA.

While HIV activation via the NF-κB pathway initiated by IκBε siRNA is attractive, it is unlikely to be the one and only HIV activation approach needed to effectively attack all of the HIV latently infected cells within the many disparate reservoirs that exist

within an HIV-infected person. Indeed, other activators that work via the NF-κB system and activators that employ other mechanisms, such as activators that act epigenetically, activate HIV in some model systems, but not in others (22, 73). Some activators can even exhibit antagonistic effects for HIV activation in some systems and additive or synergistic effects in others (22). Even NF-κB-mediated activation sometimes requires help. In some resting CD4 memory T cells, NF-κB is not sufficient for activation; activation requires both NF-κB and induction of P-TEFb (74–76).

While several different modes of attack may be required to effectively activate HIV replication for most or all HIV latently infected cells within an HIV-infected patient, our findings suggest that activation through the NF-κB pathway, achieved by specific targeting of IκBε, may make a helpful contribution. The specificity of IκBε siRNA for HIV activation and the potentially high therapeutic index of IκBε-active agents may also be enhanced by the ability to specifically target siRNAs to particular cell types. Microparticles and micelles complexed with single-chain variable fragments (scFvs) (77, 78) and aptamers (79) and other approaches are attracting increased attention as ways of directing specific siRNAs to the desired cell types (reviewed in references 80 and 81). Specific activation of HIV latently infected cells may therefore be provided by both the relative specificity for HIV activation of IκBε siRNA plus the potential to target IκBε siRNA specifically to cells likely to harbor a latent HIV provirus.

ACKNOWLEDGMENTS

We thank Tae-Wook Chun and Michael Bukrinsky for helpful discussion. IDT provided in-kind support with siRNAs and other oligonucleotides. The cell lines U1 and J-Lat 10.6 were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, through donations by Tom Folks and Eric Verdin, respectively.

This work was supported in part by the District of Columbia Developmental Center for AIDS Research (P30AI087714).

REFERENCES

1. **Havlir DV, Strain MC, Clerici M, Ignacio C, Trabattoni D, Ferrante P, Wong JK.** 2003. Productive infection maintains a dynamic steady state of residual viremia in human immunodeficiency virus type 1-infected persons treated with suppressive antiretroviral therapy for five years. J. Virol. **77**:11212–11219.
2. **Chun TW, Nickle DC, Justement JS, Large D, Semerjian A, Curlin ME, O'Shea AM, Hallahan CW, Daucher M, Ward DJ, Moir S, Mullins JI, Kovacs C, Fauci AS.** 2005. HIV-infected individuals receiving effective antiviral therapy for extended periods of time continually replenish their viral reservoir. J. Clin. Invest. **115**:3250–3255.
3. **Bisgrove D, Lewinski M, Bushman F, Verdin E.** 2005. Molecular mechanisms of HIV-1 proviral latency. Expert Rev. Anti Infect. Ther. **3**:805–814.
4. **Shen L, Siliciano RF.** 2008. Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection. J. Allergy Clin. Immunol. **122**:22–28.
5. **Geeraert L, Kraus G, Pomerantz RJ.** 2008. Hide-and-seek: the challenge of viral persistence in HIV-1 infection. Annu. Rev. Med. **59**:487–501.
6. **Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS.** 1998. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. Proc. Natl. Acad. Sci. U. S. A. **95**:8869–8873.
7. **Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, Quinn TC, Chadwick K, Margolick J, Brookmeyer R, Gallant J, Markowitz M, Ho DD, Richman DD, Siliciano RF.** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **278**:1295–1300.
8. **Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, Hermankova M, Chadwick K, Margolick J, Quinn TC, Kuo YH, Brookmeyer R, Zeiger MA, Barditch-Crovo P, Siliciano RF.** 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature **387**:183–188.
9. **Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, Smith K, Lisziewicz J, Lori F, Flexner C, Quinn TC, Chaisson RE, Rosenberg E, Walker B, Gange S, Gallant J, Siliciano RF.** 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat. Med. **5**:512–517.
10. **Siliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB, Kovacs C, Gange SJ, Siliciano RF.** 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. Nat. Med. **9**:727–728.
11. **Hamer DH.** 2004. Can HIV be cured? Mechanisms of HIV persistence and strategies to combat it. Curr. HIV Res. **2**:99–111.
12. **Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ.** 2009. The challenge of finding a cure for HIV infection. Science **323**:1304–1307.
13. **Marsden MD, Zack JA.** 2009. Eradication of HIV: current challenges and new directions. J. Antimicrob. Chemother. **63**:7–10.
14. **Mbonye U, Karn J.** 2011. Control of HIV latency by epigenetic and non-epigenetic mechanisms. Curr. HIV Res. **9**:554–567.
15. **Pion M, Jordan A, Biancotto A, Dequiedt F, Gondois-Rey F, Rondeau S, Vigne R, Hejnar J, Verdin E, Hirsch I.** 2003. Transcriptional suppression of in vitro-integrated human immunodeficiency virus type 1 does not correlate with proviral DNA methylation. J. Virol. **77**:4025–4032.
16. **Van Lint C, Emiliani S, Ott M, Verdin E.** 1996. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. EMBO J. **15**:1112–1120.
17. **Matalon S, Rasmussen TA, Dinarello CA.** 2011. Histone deacetylase inhibitors for purging HIV-1 from the latent reservoir. Mol. Med. **17**:466–472.
18. **Laughlin M, Zeichner S, Kolson D, Alwine J, Seshamma T, Pomerantz R, Gonzalez-Scaran F.** 1993. Sodium butyrate treatment of cells latently infected with HIV-1 results in the expression of unspliced viral RNA. Virology **196**:496–505.
19. **Ylisastigui L, Archin NM, Lehrman G, Bosch RJ, Margolis DM.** 2004. Coaxing HIV-1 from resting CD4 T cells: histone deacetylase inhibition allows latent viral expression. AIDS **18**:1101–1108.
20. **Archin NM, Espeseth A, Parker D, Cheema M, Hazuda D, Margolis DM.** 2009. Expression of latent HIV induced by the potent HDAC inhibitor suberoylanilide hydroxamic acid. AIDS Res. Hum. Retroviruses **25**:207–212.
21. **Contreras X, Schweneker M, Chen CS, McCune JM, Deeks SG, Martin J, Peterlin BM.** 2009. Suberoylanilide hydroxamic acid reactivates HIV from latently infected cells. J. Biol. Chem. **284**:6782–6789.
22. **Fernandez G, Zeichner SL.** 2010. Cell line-dependent variability in HIV activation employing DNMT inhibitors. Virol. J. **7**:266. doi:10.1186/1743-422X-7-266.
23. **Krishnan V, Zeichner SL.** 2004. Host cell gene expression during human immunodeficiency virus type 1 latency and reactivation and effects of targeting genes that are differentially expressed in viral latency. J. Virol. **78**:9458–9473.
24. **Tyagi M, Karn J.** 2007. CBF-1 promotes transcriptional silencing during the establishment of HIV-1 latency. EMBO J. **26**:4985–4995.
25. **Zhang Z, Klatt A, Henderson AJ, Gilmour DS.** 2007. Transcription termination factor Pcf11 limits the processivity of Pol II on an HIV provirus to repress gene expression. Genes Dev. **21**:1609–1614.
26. **du Chene I, Basyuk E, Lin YL, Triboulet R, Knezevich A, Chable-Bessia C, Mettling C, Baillat V, Reynes J, Corbeau P, Bertrand E, Marcello A, Emiliani S, Kiernan R, Benkirane M.** 2007. Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. EMBO J. **26**:424–435.
27. **Nabel G, Baltimore D.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature **326**:711–713. (Erratum, **344**:178, 1990.)
28. **Jones KA, Kadonaga JT, Luciw PA, Tjian R.** 1986. Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. Science **232**:755–759.
29. **Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ.** 1993. A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. EMBO J. **12**:3551–3558.
30. **El Kharroubi A, Piras G, Zensen R, Martin MA.** 1998. Transcriptional

activation of the integrated chromatin-associated human immunodeficiency virus type 1 promoter. Mol. Cell. Biol. **18**:2535–2544.
31. **Berkhout B, Jeang KT.** 1992. Functional roles for the TATA promoter and enhancers in basal and Tat-induced expression of the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **66**:139–149.
32. **Kaufman JD, Valandra G, Roderiquez G, Bushar G, Giri C, Norcross MA.** 1987. Phorbol ester enhances human immunodeficiency virus-promoted gene expression and acts on a repeated 10-base-pair functional enhancer element. Mol. Cell. Biol. **7**:3759–3766.
33. **Zeichner SL, Kim JY, Alwine JC.** 1991. Linker-scanning mutational analysis of the transcriptional activity of the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **65**:2436–2444.
34. **Tong-Starksen SE, Luciw PA, Peterlin BM.** 1987. Human immunodeficiency virus long terminal repeat responds to T-cell activation signals. Proc. Natl. Acad. Sci. U. S. A. **84**:6845–6849.
35. **Kinoshita S, Su L, Amano M, Timmerman LA, Kaneshima H, Nolan GP.** 1997. The T cell activation factor NF-ATc positively regulates HIV-1 replication and gene expression in T cells. Immunity **6**:235–244.
36. **Zeichner SL, Hirka G, Andrews PW, Alwine JC.** 1992. Differentiation-dependent human immunodeficiency virus long terminal repeat regulatory elements active in human teratocarcinoma cells. J. Virol. **66**:2268–2273.
37. **Basseres DS, Baldwin AS.** 2006. Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. Oncogene **25**:6817–6830.
38. **Hayden MS, Ghosh S.** 2008. Shared principles in NF-kappaB signaling. Cell **132**:344–362.
39. **Gilmore TD.** 2006. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene **25**:6680–6684.
40. **Hoffmann A, Leung TH, Baltimore D.** 2003. Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. EMBO J. **22**:5530–5539.
41. **Natoli G, Saccani S, Bosisio D, Marazzi I.** 2005. Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time. Nat. Immunol. **6**:439–445.
42. **Williams SA, Chen LF, Kwon H, Ruiz-Jarabo CM, Verdin E, Greene WC.** 2006. NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. EMBO J. **25**:139–149.
43. **Williams SA, Greene WC.** 2007. Regulation of HIV-1 latency by T-cell activation. Cytokine **39**:63–74.
44. **Williams SA, Kwon H, Chen LF, Greene WC.** 2007. Sustained induction of NF-kappa B is required for efficient expression of latent human immunodeficiency virus type 1. J. Virol. **81**:6043–6056.
45. **Rohr O, Marban C, Aunis D, Schaeffer E.** 2003. Regulation of HIV-1 gene transcription: from lymphocytes to microglial cells. J. Leukoc. Biol. **74**:736–749.
46. **Stellbrink HJ, Hufert FT, Tenner-Racz K, Lauer J, Schneider C, Albrecht H, Racz P, van Lunzen J.** 1998. Kinetics of productive and latent HIV infection in lymphatic tissue and peripheral blood during triple-drug combination therapy with or without additional interleukin-2. Antivir. Ther. **3**:209–214.
47. **Dybul M, Hidalgo B, Chun TW, Belson M, Migueles SA, Justement JS, Herpin B, Perry C, Hallahan CW, Davey RT, Metcalf JA, Connors M, Fauci AS.** 2002. Pilot study of the effects of intermittent interleukin-2 on human immunodeficiency virus (HIV)-specific immune responses in patients treated during recently acquired HIV infection. J. Infect. Dis. **185**:61–68.
48. **Chun TW, Engel D, Mizell SB, Hallahan CW, Fischette M, Park S, Davey RT, Jr, Dybul M, Kovacs JA, Metcalf JA, Mican JM, Berrey MM, Corey L, Lane HC, Fauci AS.** 1999. Effect of interleukin-2 on the pool of latently infected, resting CD4+ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. Nat. Med. **5**:651–655.
49. **Prins JM, Jurriaans S, van Praag RM, Blaak H, van Rij R, Schellekens PT, ten Berge IJ, Yong SL, Fox CH, Roos MT, de Wolf F, Goudsmit J, Schuitemaker H, Lange JM.** 1999. Immuno-activation with anti-CD3 and recombinant human IL-2 in HIV-1-infected patients on potent anti-retroviral therapy. AIDS **13**:2405–2410.
50. **Williams SA, Chen LF, Kwon H, Fenard D, Bisgrove D, Verdin E, Greene WC.** 2004. Prostratin antagonizes HIV latency by activating NF-kappaB. J. Biol. Chem. **279**:42008–42017.
51. **Korin YD, Brooks DG, Brown S, Korotzer A, Zack JA.** 2002. Effects of prostratin on T-cell activation and human immunodeficiency virus latency. J. Virol. **76**:8118–8123.
52. **Hoffmann A, Natoli G, Ghosh G.** 2006. Transcriptional regulation via the NF-kappaB signaling module. Oncogene **25**:6706–6716.
53. **Baltathakis I, Alcantara O, Boldt DH.** 2001. Expression of different NF-kappaB pathway genes in dendritic cells (DCs) or macrophages assessed by gene expression profiling. J. Cell. Biochem. **83**:281–290.
54. **Griffin BD, Moynagh PN.** 2006. In vivo binding of NF-kappaB to the IkappaBbeta promoter is insufficient for transcriptional activation. Biochem. J. **400**:115–125.
55. **Kearns JD, Basak S, Werner SL, Huang CS, Hoffmann A.** 2006. IkappaBepsilon provides negative feedback to control NF-kappaB oscillations, signaling dynamics, and inflammatory gene expression. J. Cell Biol. **173**:659–664.
56. **Nelson DE, Ihekwaba AE, Elliott M, Johnson JR, Gibney CA, Foreman BE, Nelson G, See V, Horton CA, Spiller DG, Edwards SW, McDowell HP, Unitt JF, Sullivan E, Grimley R, Benson N, Broomhead D, Kell DB, White MR.** 2004. Oscillations in NF-kappaB signaling control the dynamics of gene expression. Science **306**:704–708.
57. **Gerondakis S, Grumont R, Gugasyan R, Wong L, Isomura I, Ho W, Banerjee A.** 2006. Unravelling the complexities of the NF-kappaB signalling pathway using mouse knockout and transgenic models. Oncogene **25**:6781–6799.
58. **Pasparakis M, Luedde T, Schmidt-Supprian M.** 2006. Dissection of the NF-kappaB signalling cascade in transgenic and knockout mice. Cell Death Differ. **13**:861–872.
59. **Klement JF, Rice NR, Car BD, Abbondanzo SJ, Powers GD, Bhatt PH, Chen CH, Rosen CA, Stewart CL.** 1996. IkappaBalpha deficiency results in a sustained NF-kappaB response and severe widespread dermatitis in mice. Mol. Cell. Biol. **16**:2341–2349.
60. **Thompson JE, Phillips RJ, Erdjument-Bromage H, Tempst P, Ghosh S.** 1995. I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. Cell **80**:573–582.
61. **Whiteside ST, Epinat JC, Rice NR, Israel A.** 1997. I kappa B epsilon, a novel member of the I kappa B family, controls RelA and cRel NF-kappa B activity. EMBO J. **16**:1413–1426.
62. **Li Z, Nabel GJ.** 1997. A new member of the I kappaB protein family, I kappaB epsilon, inhibits RelA (p65)-mediated NF-kappaB transcription. Mol. Cell. Biol. **17**:6184–6190.
63. **Memet S, Laouini D, Epinat JC, Whiteside ST, Goudeau B, Philpott D, Kayal S, Sansonetti PJ, Berche P, Kanellopoulos J, Israel A.** 1999. IkappaBepsilon-deficient mice: reduction of one T cell precursor subspecies and enhanced Ig isotype switching and cytokine synthesis. J. Immunol. **163**:5994–6005.
64. **Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS.** 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science **238**:800–802.
65. **Jordan A, Bisgrove D, Verdin E.** 2003. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J. **22**:1868–1877.
66. **Colin L, Van Lint C.** 2009. Molecular control of HIV-1 postintegration latency: implications for the development of new therapeutic strategies. Retrovirology **6**:111. doi:10.1186/1742-4690-6-111.
67. **Le Douce V, Janossy A, Hallay H, Ali S, Riclet R, Rohr O, Schwartz C.** 2012. Achieving a cure for HIV infection: do we have reasons to be optimistic? J. Antimicrob. Chemother. **67**:1063–1074.
68. **Marsden MD, Zack JA.** 2010. Establishment and maintenance of HIV latency: model systems and opportunities for intervention. Future Virol. **5**:97–109.
69. **Malek S, Chen Y, Huxford T, Ghosh G.** 2001. IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. J. Biol. Chem. **276**:45225–45235.
70. **Tam WF, Sen R.** 2001. IkappaB family members function by different mechanisms. J. Biol. Chem. **276**:7701–7704.
71. **Kanarek N, Ben-Neriah Y.** 2012. Regulation of NF-kappaB by ubiquitination and degradation of the IkappaBs. Immunol. Rev. **246**:77–94.
72. **Tran K, Merika M, Thanos D.** 1997. Distinct functional properties of IkappaB alpha and IkappaB beta. Mol. Cell. Biol. **17**:5386–5399.
73. **McNamara LA, Ganesh JA, Collins KL.** 2012. Latent HIV-1 infection occurs in multiple subsets of hematopoietic progenitor cells and is reversed by NF-kappaB activation. J. Virol. **86**:9337–9350.
74. **Lassen KG, Hebbeler AM, Bhattacharyya D, Lobritz MA, Greene WC.**

2012. A flexible model of HIV-1 latency permitting evaluation of many primary CD4 T-cell reservoirs. PLoS One **7**:e30176. doi:10.1371/journal.pone.0030176.

75. **Tyagi M, Pearson RJ, Karn J.** 2010. Establishment of HIV latency in primary $CD4^+$ cells is due to epigenetic transcriptional silencing and P-TEFb restriction. J. Virol. **84**:6425–6437.
76. **Yang HC, Xing S, Shan L, O'Connell K, Dinoso J, Shen A, Zhou Y, Shrum CK, Han Y, Liu JO, Zhang H, Margolick JB, Siliciano RF.** 2009. Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. J. Clin. Invest. **119**:3473–3486.
77. **Kumar P, Ban HS, Kim SS, Wu H, Pearson T, Greiner DL, Laouar A, Yao J, Haridas V, Habiro K, Yang YG, Jeong JH, Lee KY, Kim YH, Kim SW, Peipp M, Fey GH, Manjunath N, Shultz LD, Lee SK, Shankar P.** 2008. T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. Cell **134**:577–586.
78. **Kim SS, Subramanya S, Peer D, Shimaoka M, Shankar P.** 2011. Antibody-mediated delivery of siRNAs for anti-HIV therapy. Methods Mol. Biol. **721**:339–353.
79. **Wheeler LA, Trifonova R, Vrbanac V, Basar E, McKernan S, Xu Z, Seung E, Deruaz M, Dudek T, Einarsson JI, Yang L, Allen TM, Luster AD, Tager AM, Dykxhoorn DM, Lieberman J.** 2011. Inhibition of HIV transmission in human cervicovaginal explants and humanized mice using CD4 aptamer-siRNA chimeras. J. Clin. Invest. **121**:2401–2412.
80. **de Fougerolles AR.** 2008. Delivery vehicles for small interfering RNA in vivo. Hum. Gene Ther. **19**:125–132.
81. **Peer D, Lieberman J.** 2011. Special delivery: targeted therapy with small RNAs. Gene Ther. **18**:1127–1133.