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Potent and Stable Attenuation of Live-HIV-1 by Gain of a Proteolysis-resistant Inhibitor of NF-κB (IκB-αS32/36A) and the Implications for Vaccine Development*

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Live-attenuated human immunodeficiency viruses (HIVs) are candidates for Acquired Immunodeficiency Syndrome (AIDS) vaccine. Based on the simian immunodeficiency virus (SIV) model for AIDS, loss-of-function (*e.g.* deletion of accessory genes such as *nef*) has been forwarded as a primary approach for creating enfeebled, but replication-competent, HIV-1/SIV. Regrettably, recent evidence suggests that loss-of-function alone is not always sufficient to prevent the emergence of virulent mutants. New strategies that attenuate via mechanisms distinct from loss-of-function are needed for enhancing the safety phenotype of viral genome. Here, we propose gain-of-function to be used simultaneously with loss-of-function as a novel approach for attenuating HIV-1. We have constructed an HIV-1 genome carrying the cDNA of a proteolysis-resistant nuclear factor-κB inhibitor (IκB-αS32/36A) in the *nef* region. HIV-1 expressing IκB-αS32/36A down-regulates viral expression and is highly attenuated in both Jurkat and peripheral blood mononuclear cells. We provide formal proof that the phenotypic and attenuating characteristics of IκB-αS32/36A permit its stable maintenance in a live, replicating HIV-1 despite 180 days of forced *ex vivo* passaging in tissue culture. As compared with other open-reading frames embedded into HIV/SIV genome, this degree of stability is unprecedented. Thus, IκB-αS32/36A offers proof-of-principle that artifactually gained functions, when used to attenuate the replication of live HIV-1, can be stable. These findings illustrate gain-of-function as a feasible strategy for developing safer live-attenuated HIVs to be tested as candidates for AIDS vaccine.

Live-attenuated human immunodeficiency virus, type-1 (HIV-1)[1] viruses are vaccine candidates for AIDS based on the assumption that poorly replicating viruses are reduced in cytopathicity while eliciting an efficient immune response *in vivo*. In fact, in the SIV model of AIDS, viruses deleted of accessory genes, such as *nef*, are attenuated and confer immune protection in adults (1–6). Recent results have raised concerns about the safety of such live-attenuated viruses because of the emergence of virulent mutants (7–11). Hence, it is reasonable that new mechanistically distinct strategies should be considered in combination with loss-of-function to develop safe live-attenuated vaccine candidates. Stable attenuation of lentivirus is difficult. Reasons for this include the high mutability of retroviruses as a consequence of poor fidelity in reverse transcription and high rates of viral replication. Virus replication is one of several parameters used to measure HIV disease progression. To this end, endowing the viral genome with an inhibitory function that represses replication is, in principle, a valid strategy for attenuation.

AIDS pathogenesis involves complex host-pathogen interactions (12–17). HIV-1 entry occurs through binding of gp120 envelope glycoprotein to CD4 and chemokine co-receptors (18, 19). Later, after reverse transcription and integration, regulated HIV-1 gene expression is dependent on cellular transcription factors and on the viral Tat protein acting through several *cis* regulatory sequences in the HIV-1 LTR (20, 21). Prominent among these sequences are two NF-κB sites located upstream of the Sp1-TATAA motif (22) and a third NF-κB site co-incident with the TAR sequence (23). The prevalence of NF-κB sites in all HIV isolates (24) suggests that NF-κB function is fundamentally important for virus replication and represents an attractive target to attenuate HIVs. NF-κB is regulated by IκB inhibitors (25). In response to activating stimuli, IκB proteins become phosphorylated, ubiquinated, and degraded by proteasomes. This releases active NF-κB complexes leading to transcriptional activation of responsive genes. An IκB-α mutant protein, IκB-αS32/36A (defective for serine 32- and serine 36-phosphorylation), was previously shown to resist proteolysis (26, 27). IκB-αS32/36A was found to be a particularly potent inhibitor of NF-κB-dependent gene transcription (26, 27). Based on the role of NF-κB in virus transcription, we reasoned that expression of IκB-αS32/36A could be one way to attenuate HIV-1.

Here, we have investigated the feasibility of a stable bimodal attenuation of HIV-1 through both gain- and loss-of-function.

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus, type-1; SIV, simian immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; LTR, long terminal repeat; NF-κB, nuclear factor kappa B; RT, reverse transcriptase; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell; CAT, chloramphenicol acetyltransferase; PHA, phytohemagglutinin; nt, nucleotide(s); bp, base pair(s); mAb, monoclonal antibody.

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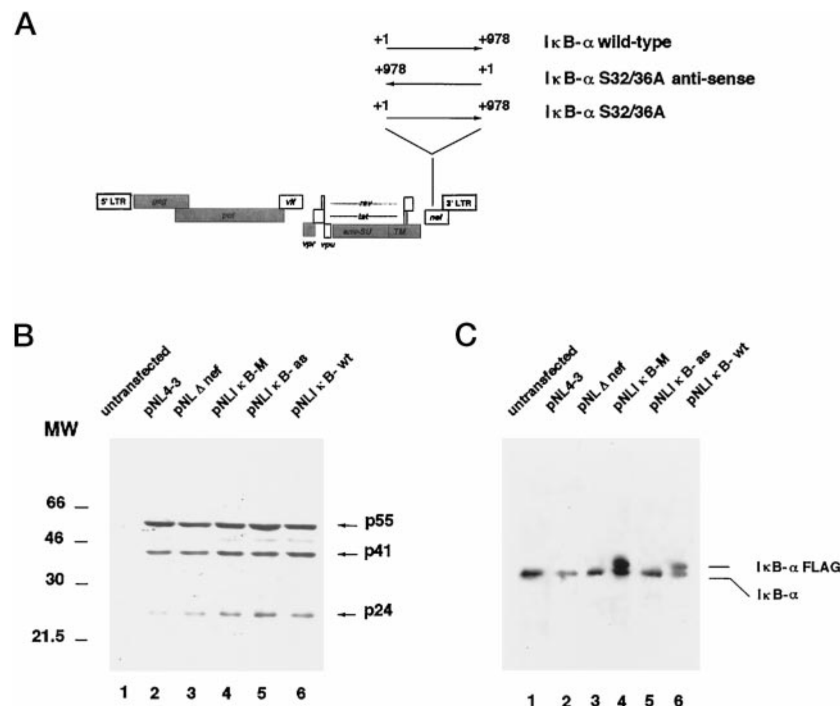

FIG. 1. **Recombinant HIV-1 viruses that express IκB-α.** *Panel A*, structure of HIV-1 genomes that express IκB-α wild-type (pNLIκB-wt), IκB-αS32/36A in sense (pNLIκB-M), and antisense (pNLIκB-as) orientations, respectively. *Panel B*, immunoblot analysis of total extracts (10 μg each) from 293T cells 24 h after transfection with the indicated HIV-1 plasmids (10 μg) using a hyperimmune AIDS patient serum. *Panel C*, immunoblot analysis of total extracts (10 μg) from 293T cells 24 h after transfection with the indicated HIV-1 plasmids (10 μg) using anti-FLAG monoclonal antibody followed by an anti-IκB-α antiserum. This mixture identifies both IκB-α-FLAG expressed from the viral genomes and endogenous IκB-α.

We show that a proteolysis-resistant dominant negative IκB-α molecule (IκB-αS32/36A) can be inserted into *nef* of HIV-1, is maintained in the viral genome despite prolonged passaging in tissue culture, and contributes to a strong attenuation of HIV-1. These results indicate that gain-of-function should be considered together with loss-of-function in strategies for constructing safe live-attenuated HIV-1 vaccine candidates. In addition, they demonstrate that NF-κB inhibition is a major target for HIV-1 attenuation.

EXPERIMENTAL PROCEDURES

Construction of IκB-α Recombinant HIV-1 Viruses—pNLΔnef was generated by replacing the first 117 nucleotides of *nef* sequence of pNL4–3 (28) with a linker specifying for the unique restriction sites *Xho*I, *Xba*I, and *Not*I. This resulted in the frameshift of the remaining *nef* sequence. cDNAs for IκB-α and IκB-αS32/36A were amplified by PCR using a 5′-primer flanked by *Sal*I site and a 3′-primer flanked by the FLAG sequence followed by stop codon and *Xba*I site. The 5′-primer was 5′-ACGCGTCGACATGTTCCAGGCGGCCGAGCGC-3′; the 3′-primer was 5′-GCTCTAGATCACTTGTCGTCATCGTCTTTGTAGTCTAACGTCAGACGCTGGCCTCCAAA-3′. The PCR products for IκB-α and IκB-αS32/36A were digested with *Sal*I and *Xba*I and ligated into pNLΔnef to generate pNLIκB-wt and pNLIκB-M, respectively. IκB-αS32/36A was also cloned in antisense orientation using a 5′-primer flanked by *Xba*I site and a 3′-primer flanked by *Sal*I site to generate pNLIκB-as. Molecular clones were confirmed by DNA restriction analysis and DNA sequencing.

Transfections and Viral Stocks—293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% v/v heat-inactivated fetal bovine serum and 3 mM glutamine. Viral stocks were produced by transfecting 293T cells (5×10^6) with viral plasmids (10 μg) using calcium phosphate. Forty hours later, the cell culture supernatant was passed through a 0.45-μm filter and measured for RT activity.

Immunoblot Analysis—293T cells were transfected with viral plasmids (10 μg) and were lysed in RIPA buffer 24 h later. Proteins (10 μg) were separated by electrophoresis in 10% SDS-polyacrylamide gel and transferred to Immobilon-P (Millipore). Filters were blotted with AIDS patient serum or with anti-FLAG monoclonal antibody (Eastman Kodak, Rochester, NY) and anti-IκB-α antiserum recognizing the amino acids 51–64 of IκB-α (gift from N. Rice, National Cancer Institute-Frederick Cancer Research and Development Center, MD) using Western-Light Chemiluminescent Detection System (Tropix, Bedford, MA).

RNase Protection Assay—293T cells were transfected with viral plasmids (10 μg) using calcium phosphate, and total RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction 3 days after transfection. RNA was treated with RQ1 DNase from Promega (2 units/50 μg of RNA) at 37 °C for 15 min, extracted by phenol:chloroform (25:24) and precipitated in ethanol. RNase protection assay was performed using RPA II kit (Ambion, Austin, TX). Briefly, aliquots of 10 μg of RNA from different samples were hybridized with 10^5 cpm of HIV-1 and actin RNA probes for 15 h at 44 °C. Yeast RNA was used as control. Samples were treated with RNase A (2.5 units/ml) and RNase T1 (100 units/ml) for 30 min at 37 °C, precipitated in ethanol, resuspended in gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromphenol blue, 0.5 mM EDTA, 0.025% SDS), and separated by electrophoresis on 8 M urea, 8% acrylamide gel in Tris borate-EDTA. HIV-1 antisense probe to the pNL4–3 sequence from +350 to +535 nt corresponding to NF-κB, Sp1, TATAA, and TAR sequences of HIV-1 LTR was transcribed from the T7 promoter in the pGEM LTR vector. Protected HIV-1-specific bands of 80 and 185 bp correspond to the 5′-LTR (+455 to +535 nt of pNL4–3 sequence) and the 3′-LTR (+9425 to +9610 nt of pNL4–3 sequence), respectively. Actin antisense probe was transcribed using T7 RNA-polymerase from vector pTRI-β-actin-125-human (Ambion). Protected actin transcript corresponds to a band of 127 bp. T7 RNA polymerase-derived transcripts were produced by using the Riboprobe *in vitro* Transcription System (Promega). Transfection efficiencies were normalized to CAT activity measured from co-transfections with pBLCAT2 (2 μg), which contains the *cat* gene fused to the herpes simplex virus *tk* minimal promoter, followed by CAT assay (23).

Luciferase Assay—Jurkat cells (2×10^6) were co-transfected with pNL-Luc-R$^-$E$^-$ plasmid (29) (1 μg) and pNLIκB-as or pNLIκB-M plasmids (5 μg) using Superfect Reagent (Qiagen, Valencia, CA). Amounts of transfected DNA were equalized with pcDNA3.1 (Stratagene, La Jolla, CA). Transfected cells were cultured in RPMI supplemented with 10% v/v heat-inactivated fetal bovine serum and 3 mM glutamine with soluble CD4 (10 μg/ml) to inhibit multiple rounds of infection. Cell extracts were prepared at 24, 48, and 72 h post-transfection and analyzed for luciferase activity using Luciferase Assay System (Promega).

Viral Growth—Jurkat cells were cultured in RPMI supplemented with 10% v/v heat-inactivated fetal bovine serum and 3 mM glutamine. PBMCs were stimulated with PHA (0.5 μg/ml) or mAb OKT3 (1 μg/ml) in RPMI 1640 with 10% v/v heat-inactivated fetal bovine serum for 3 days and then washed and cultured in RPMI 1640 with 10% fetal bovine serum with interleukin-2 (20 units/ml). Cells were infected with equivalent doses of viral stocks normalized by RT activity. Cell supernatants were collected every 3 days for RT assay; equal volumes of fresh medium were replaced into the cultures at the same time.

RT-PCR Analysis—Viral RNA was purified from cell culture supernatants using QIAampViral RNA kit (Qiagen). RNA extracts (1 μg) were reverse transcribed, and PCR was amplified using Titan One Tube RT-PCR System (Roche Molecular Biochemicals). The IκB-αS32/36A sequence was amplified with primers flanking the cDNA insert from +8732 to +8749 nt (5′-primer; *env* region) and from +9017 to +9034 nt

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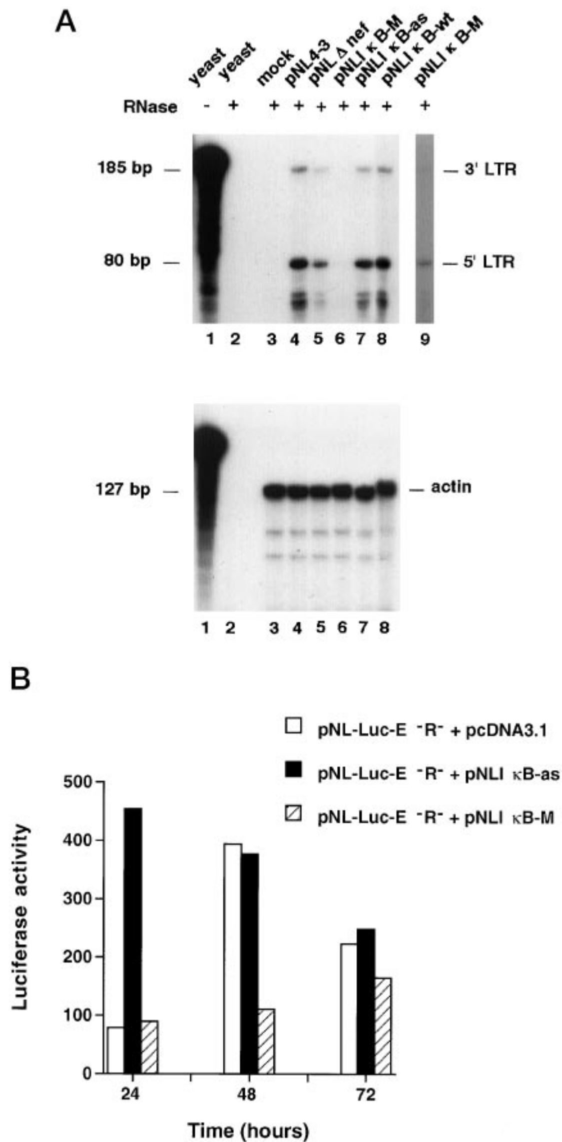

FIG. 2. **IκB-αS32/36A represses HIV-1 LTR-directed transcription.** *Panel A*, 293T cells were transfected with the indicated viral plasmids (10 μg). Transfection efficiencies were normalized based on the co-transfection with 2 μg of pBLCAT2. Total RNAs were quantitated by RNase protection assay 72 h after transfection. Viral RNAs were measured as 3′-LTR and 5′-LTR protected fragments. Cellular actin-mRNA was quantitated in parallel. *Lane 1*, RNA probe plus yeast RNA; *lane 2*, RNase treatment of RNA probe plus yeast RNA; *lane 3*, RNase treatment of RNA probe plus RNA from cells transfected only with pBLCAT2. *Lanes 4–8*, RNase treatment of RNA probe plus RNA from cells transfected with pNL4–3 (*lane 4*), pNLΔnef (*lane 5*), pNLIκB-M (*lane 6*), pNLIκB-as (*lane 7*), and pNLIκB-wt (*lane 8*). *Lane 9*, sample as in *lane 6* with a 10-fold longer exposure. CAT activities, measured as percent acetylation of [^{14}C]chloramphenicol per 50 μg of protein per 3 h, from *lane 3–8* were: 1.5, 1.7, 1.0, 1.6, 1.2, and 1.9, respectively. Results are representative of three independent experiments. *Panel B*, Jurkat cells were transfected with pcDNA3.1, pNLIκB-as, or pNLIκB-M plasmids (5 μg) together with pNL-Luc-R$^-$E$^-$ reporter plasmid (1 μg). Luciferase assay was performed in cell lysates at the indicated time. Luciferase activity is expressed as arbitrary light units per 100 μg of protein. Results are representative of three independent experiments.

(3′-primer; *nef* region) of pNL4–3. As control, the integrase region was amplified with 5′-primer from +4339 to +4359 nt and 3′-primer from +4894 to +4914 nt of pNL4–3. Amplification was carried out according to the following protocol: incubation at 50 °C for 30 min, preheating at 95 °C for 2 min followed by 30 cycles of 95 °C for 45 s, 58 °C for 45 s, and 72 °C for 3 min.

Flow Cytometry Analysis—Cells were stained with phycoerythrin-conjugated anti-p24 mouse monoclonal antibody (Ortho Diagnostic, Raritan, NJ) and Annexin-V-Fluos (Roche Molecular Biochemicals). Flow cytometry was performed with a Coulter Epics XL Cytometer. Ten thousands cells were analyzed under each condition.

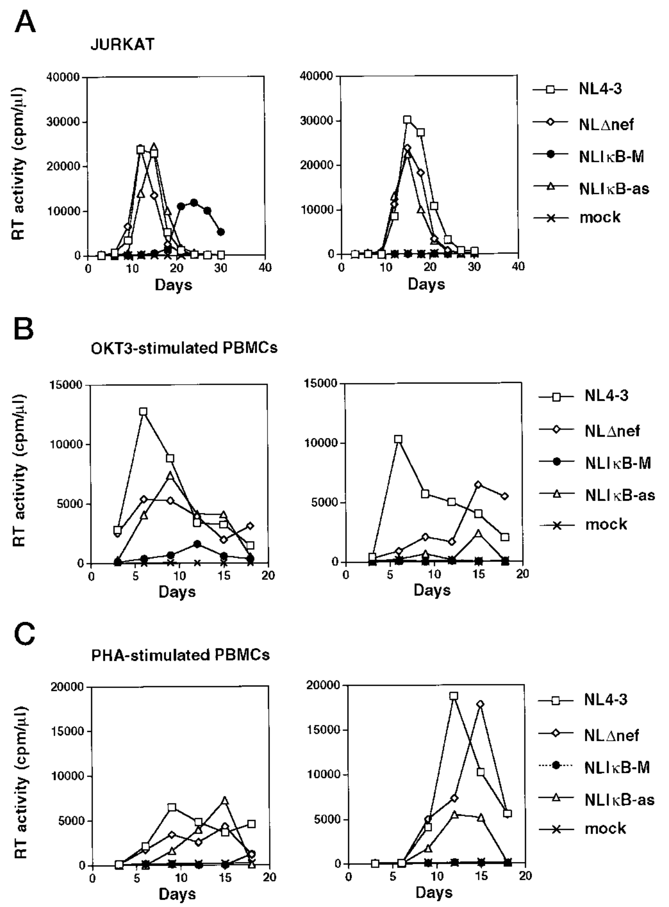

FIG. 3. **HIV-1 expressing IκB-αS32/36A is highly attenuated in Jurkat cells and human PBMCs.** *Panel A*, Jurkat (5 × 10^4) cells were infected with NL4–3, NLΔnef, NLIκB-M, and NLIκB-as with equal amounts of viruses normalized based on RT counts of 10^4 cpm (*left*) or 10^3 cpm (*right*). *Panel B*, PBMCs (2 × 10^5 cells) stimulated with mAb OKT3 were infected with the indicated viruses normalized for 10^5 cpm (*left*) or 10^4 cpm (*right*) of RT. *Panel C*, PBMCs (2 × 10^5 cells) stimulated with PHA were infected with viruses normalized for 10^4 cpm (*left*) or 10^3 cpm (*right*) of RT. Viral growth was measured by RT activity in cell culture supernatants. Results representative of six independent infections are shown.

RESULTS

HIV-1 Expressing IκB-αS32/36A Down-regulates Viral Expression—To determine whether the attenuation of HIV-1 could be obtained via *cis*-expression of a proteolysis-resistant inhibitor of NF-κB, FLAG-tagged IκB-αS32/36A cDNA was positioned in sense and antisense orientations into *nef* of HIV-1 pNL4–3 generating pNLIκB-M and pNLIκB-as genomes, respectively (Fig. 1*A*). As a control, an otherwise isogenic molecular clone of pNL4–3 expressing the proteolysis-sensitive wild-type IκB-α (pNLIκB-wt) cDNA was also constructed (Fig. 1*A*). These insertions also resulted in the deletion of 39 amino acids from the N terminus of Nef and a translational frameshift for the remaining Nef-encoding codons. Hence, the chimeric genomes gained an IκB-αS32/36A function coupled with a simultaneous loss of Nef function. From these molecular genomes, different virus stocks were generated through independent transfections of 293T cells. Expression in the transfected cells of HIV-1 proteins and the heterologous IκB-cDNAs was verified by immunoblotting (Fig. 1, *B* and *C*). To assess the functional impact of IκB-αS32/36A in the context of HIV-1 gene expression, viral transcription in 293T cells transfected individually

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with pNL4–3, pNLΔnef, pNLIκB-M, pNLIκB-as, or pNLIκB-wt was analyzed by RNase protection assays. Viral RNAs were efficiently produced from all genomes except for the IκB-αS32/36A-expressing virus, pNLIκB-M (Fig. 2*A*). This finding points to a strong inhibition of LTR-directed transcription by IκB-αS32/36A. A normalized simultaneous co-transfection into cells of pNLIκB-M genome with pNL4–3 genome fused to the *luciferase* reporter gene still resulted in markedly reduced viral transcription (Fig. 2*B*). The inhibitory mechanism is likely to be a dominant *trans*-repressive effect from the IκB-αS32/36A protein rather than a *cis*-destabilizing phenomenon arising as a consequence of an insertion into *nef*. This is further supported by the fact that the insertion of IκB-α cDNA either in antisense orientation, or as wild-type sequence does not inhibit viral expression (Fig. 2*A*, *lanes 7* and *8*). Repressed expression would have been expected if there were a *cis* insertional effect.

Attenuation of HIV-1 Expressing IκB-αS32/36A—Next, the replication properties of the recombinant HIV-1 genomes were measured. We monitored for viral growths in established (Jurkat) and primary (PBMCs) T-cells. Based on normalized amounts of input viruses, NLIκB-M was found to be highly attenuated in Jurkat cells when compared with the NLΔnef and NLIκB-as (Fig. 3*A*). A reduced replication capacity was also observed for NLIκB-M in human PBMCs stimulated either with anti-CD3 mAb OKT3 (Fig. 3*B*) or PHA (Fig. 3*C*). In parallel, the chimeric HIV-1 which expresses the proteolysis-sensitive wild-type IκB-α produced a replication profile that was only slightly attenuated when compared with control NLIκB-as virus (Fig. 4, *A–C*). Taken together, these results indicate a critical contribution of proteolysis-resistant mutant IκB-α toward attenuated viral growth.

Attenuation of NLIκB-M Is Not Because of Apoptosis—Previously, IκB-αS32/36A was shown to enhance the apoptosis induced by tumor necrosis factor-α (30–32). Because tumor necrosis factor-α is produced during HIV-1 infection (33, 34), we asked whether a pro-apoptotic effect of IκB-αS32/36A might account, in part, for attenuation of viral growth. To this end, Jurkat cells infected separately with NL4–3, NLIκB-M, or NLIκB-as viruses were analyzed by flow cytometry for the expression of p24 and for binding to annexin V, a marker of an early event in apoptosis (35). Because of the different viral growth kinetics, to account fully for equivalent levels of virus production, cell samples were analyzed at variant times post-infection. As shown in Table I, the percentage of apoptotic cells among p24-positive population was 17 and 13% for NL4–3 and NLIκB-as, respectively, at day 10 post-infection, as compared with 19% for NLIκB-M at day 18 post-infection. Thus, the expression of IκBαS32/36A did not affect apoptosis significantly because the percentage of apoptotic cells among those infected with NL4–3, NLIκB-as, or NLIκB-M was similar.

NLIκB-M Is Stably Attenuated through Cell Passaging—Retroviruses rapidly delete heterologous genes which do not confer benefits for replication (36–38). One indication of such a loss in gained function would be a change in the kinetics of replication in later (compared with earlier) passages of viruses. In examining NLIκB-M virus after prolonged culturing in cells, we noted that its attenuated phenotype bred true despite extended re-passaging (Fig. 5, *A–C*). No changes in replication phenotype were observed with up to 180 days (9 passages) of continuously forced propagation in *ex vivo* tissue cultures. This stability of IκB-αS32/36A contrasts sharply with documented findings in other systems where rapid deletion of gained func-

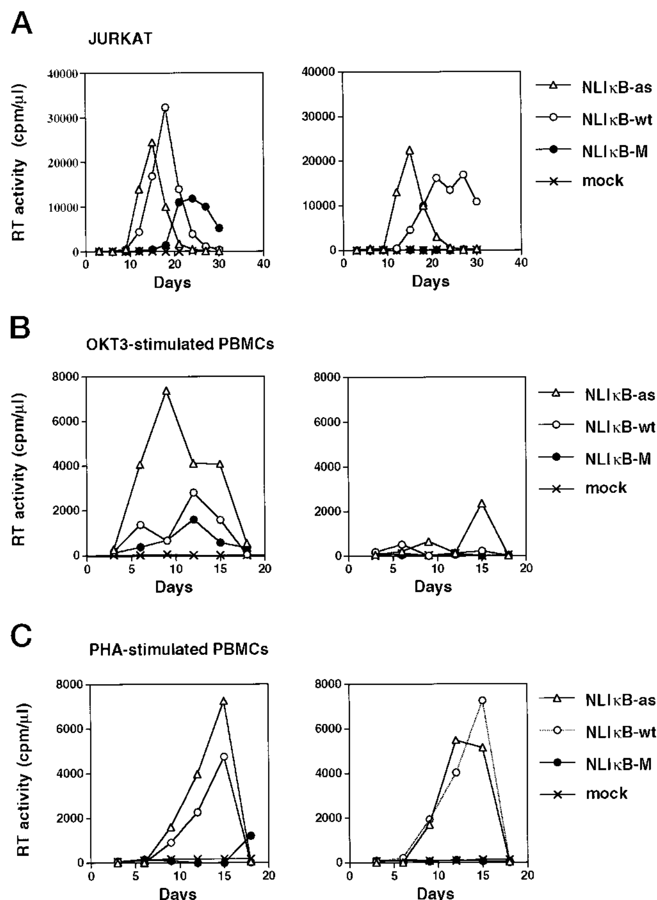

FIG. 4. **Wild-type proteolysis-sensitive IκB-α poorly attenuates HIV-1 replication.** Jurkat cells (*panel A*), PBMCs stimulated with mAb OKT3 (*panel B*), and PBMCs stimulated with PHA (*panel C*) were infected with NLIκB-M, NLIκB-as, or NLIκB-wt viruses and measured for viral production as detailed in Fig. 3.

TABLE I
Flow cytometric analysis of Jurkat cells infected with NL4–3, NLIκB-as, or NLIκB-M

The cells were analysed by flow cytometry for the expression of p24, as marker of viral infection, and for binding to annexin V, as marker of apoptosis. Ten thousand cells were analysed under each condition. Values are expressed as percentages.

Cells	p24+		p24−	
	Annexin V−	Annexin V+	Annexin V−	Annexin V+
Day 10 post-infection				
NL4–3 infected	77	16	4	3
NLIκB-as infected	68	10	18	4
NLIκB-M infected	3	1	83	13
Uninfected	0.4	0.5	91	8.1
Day 18 post-infection				
NL4–3 infected	8	69	1	22
NLIκB-as infected	5	52	4	39
NLIκB-M infected	19	4.5	63	13.5
Uninfected	1.4	1.6	84	13

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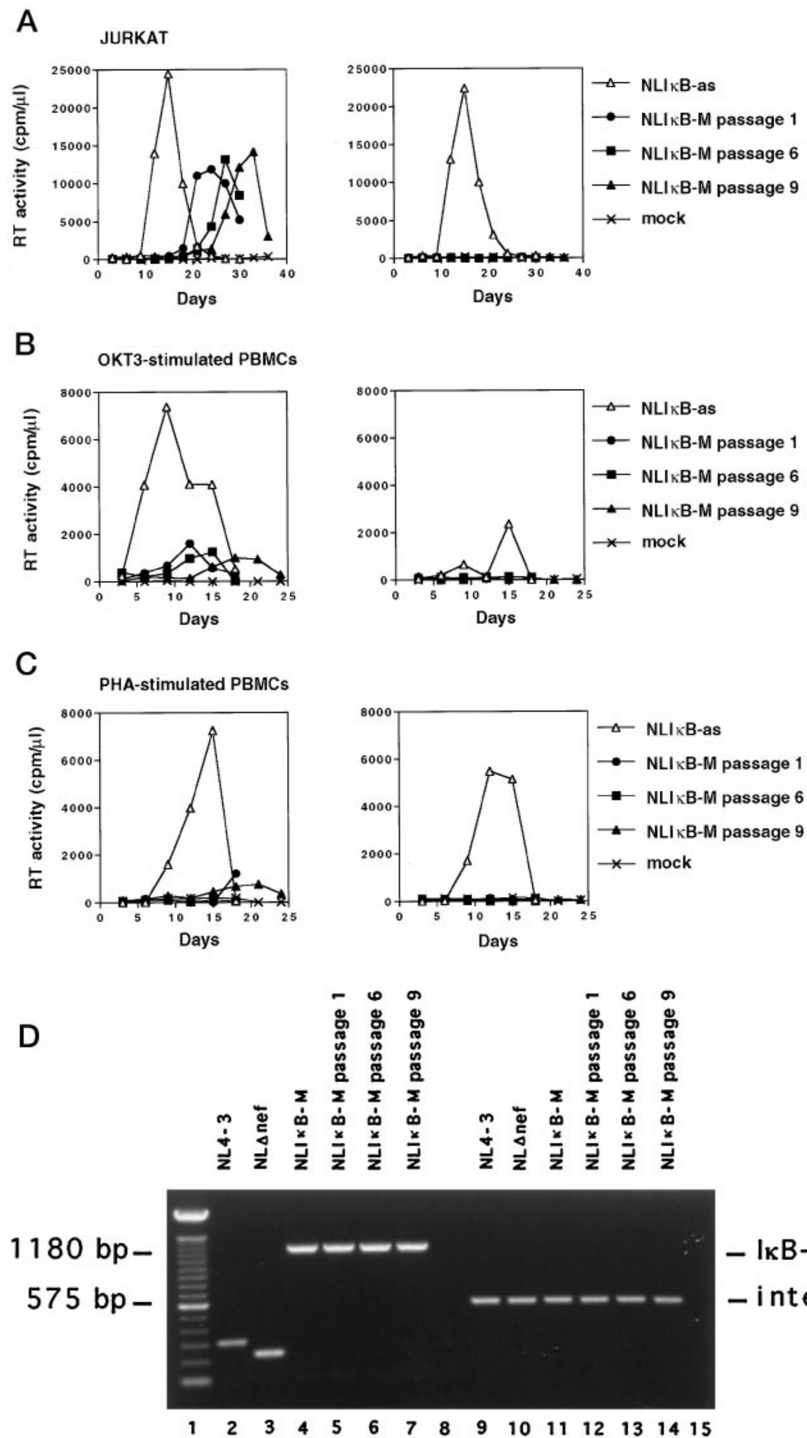

FIG. 5. **NLIκB-M virus maintains an attenuated phenotype through serial passaging.** Jurkat (*panel A*), PBMCs stimulated with mAb OKT3 (*panel B*), and PBMCs stimulated with PHA (*panel C*) were infected with NLIκB-as, NLIκB-M, or passaged NLIκB-M viruses and measured for viral production as detailed in Fig. 3. NLIκB-M virus was obtained through serial passages in Jurkat cells by collecting the RT peak of viral production at each passage. On average, each passage was for 20 days. *Panel D*, RT-PCR analysis of *nef* and *integrase* regions of NLIκB-M virion RNAs. The wild-type *nef* product (302 bp; *lane 2*), the Δnef product (200 bp; *lane 3*), the IκB-αS32/36A product (1180 bp; *lanes 4–7*), and the integrase product (575 bp; *lanes 9–14*) were visualized by RT-PCR of viral RNA. *Lane 1*, 100-bp DNA ladder (Life Technologies, Inc.); *lanes 2* and *9*, amplification of NL4–3 virion RNAs from 293T transfected with pNL4–3. *Lanes 3* and *10*, amplification of NLΔnef virion RNAs from 293T transfected with pNLΔnef; *lanes 4* and *11*, amplification of NLIκB-M virion RNAs from 293T transfected with pNLIκB-M; *lanes 5* and *12*, amplification of NLIκB-M virion RNAs from first passage in Jurkat; *lanes 6* and *13*, amplification of NLIκB-M virion RNAs from sixth passage in Jurkat; *lanes 7* and *14*, amplification of NLIκB-M virion RNAs from ninth passage in Jurkat; *lanes 8* and *15*, RT-negative controls.

tion commenced as early as 48 h after viral passaging (36). To verify biochemically that this phenotypic stability was from the maintenance of IκB-αS32/36A in *nef*, RT-PCR analysis of virion RNAs was performed using samples isolated after various times of culturing in Jurkat cells (Fig. 5*D*). Viral RNAs from first passage (20 days in culture) as compared with sixth (120 days in culture) and ninth (180 days in culture) passage were indistinguishable, providing no evidence for deletion of IκB-αS32/36A (Fig. 5*D*, compare *lanes 5–7*). Thus, by contrast with other cDNAs inserted into *nef* (36–38) in the setting of replicating HIV-1/SIV, IκB-αS32/36A is unprecedently stable.

DISCUSSION

Various approaches have been proposed for generating live-attenuated HIV-1 (39). Based on findings from the SIV model of AIDS, loss-of-function (*e.g.* deletion of accessory genes such as *nef*, *vpr,* and U3) has been forwarded as a primary approach for creating enfeebled, but replication-competent, HIV/SIV (1–6). Regrettably, recent evidence indicates that loss-of-function alone is not always sufficient (and is perhaps largely insufficient) to prevent virulence (7–11). Hence, one is faced with either abandoning live-attenuated virus as a potential route for HIV-1 vaccine or devising another approach which is mechanistically distinct from and could be used additively with loss-of-function in attenuating the AIDS virus. Attenuation of HIV-1 by gain-of-function was first broached four years ago (40). However, an issue that has impeded the useful application of gain-of-function is uncertainty over the stability of functions added to a replicating HIV-1 genome. To date, all exogenous

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open-reading frames, such as thymidine kinase, γ-interferon, interleukin-2 (36–38), when artifactually embedded into the HIV-1/SIV genome have been unstable to prolonged *ex vivo* passaging, suggesting a rule of rapid deletion of gained genes upon virus replication. However, because the sampling size for gained functions has been small, it remains unclear whether this is indeed a universal rule. Our findings from IκB-αS32/36A formally challenge the rigidity of this rule.

An ability by viruses in general and by HIV-1 specifically to maintain stably gained functions should not be inherently surprising. For example, piracy of large numbers of cellular genes by herpesviruses has been well documented (see Ref. 41 and references cited therein). In simple animal retroviruses, only Gag, Pol, and Env open-reading frames are required for virus propagation. Hence, the evolution of *tat, rev, nef, vpu, vif*, and *vpr* in the existing HIV-1 genome represents likely illustrations of how this virus has anciently through natural means acquired and continues to maintain stably added reading frames. Conceivably, IκB-αS32/36A has certain cellular characteristics that benefit the virus-cell interaction during HIV-1 replication. What might be the propitious characteristics specified by IκB-αS32/36A await further investigation. It is possible that the inhibition of virus transcription by IκB-αS32/36A allows the cell to survive HIV-1 infection, while cell killing occurs in the case of recombinant viruses that carry exogenous genes which do not confer virus attenuation. A second possible explanation for the insert stability in NLIκB-M is that IκB-αS32/36A could provide an "anti-mutator" effect to the chimeric HIV-1 genome. This "anti-mutator" effect would confer a relative resistance to the deletion of the gained function. In this regard, the expression of a dominant negative IκB-α has been shown to correct aberrant DNA synthesis which causes genetic instability in ataxia telangectasia cells (42).

In conclusion, we demonstrate here the feasibility of a gain-of-function strategy for stably attenuating live HIV-1. Specifically, we show that the addition of IκB-αS32/36A to an HIV-1 genome conferred a highly attenuated replication phenotype that is genetically stable for a minimum of 180 days of growth in tissue culture. Attenuated replication in *ex vivo* tissue culture is by no means a perfect predictor of *in vivo* viral robustness; however, the unprecedented observation that emerges from IκB-αS32/36A is its durability in settings where other artifactually gained open-reading frames (36–38) have failed. An important extension to the experiments here would be to explore the *in vivo* stability of IκB-αS32/36A in chimeric SIVs propagated in macaques. However, the fact that transcription from the SIV LTR is not ruled by NF-κB in the same manner as that from the HIV-1 LTR (43–45) undermines the feasibility of such a heterologous *in vivo* test for IκB-αS32/36A. In this regard, further studies on SIV LTR-driven transcription are warranted to identify analogous cellular inhibitors of SIV replication. That not withstanding, the findings from IκB-αS32/36A represent a first-step proof that gain-of-function could be a stably useful approach for attenuation. This first step should spur further stepwise exploration for other useful attenuating markers that might be more amenable to incremental testing in nonhuman primate models. If so, a combination approach that incorporates gain-of-function with loss-of-function could possibly contribute toward the development of a live-attenuated HIV-1 vaccine.

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